SYSTEMIC CONTROL OF SOYBEAN NODULE AUTOREGULATION

by

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DECLARATION

The research described in this thesis is my own work, except where acknowledgement is made, and has not been submitted for any other degree. Dr. Peter Gresshoff assisted in the planning of experiments in chapters 3, 4 and 5 and Dr. Angela Delves assisted with the planning of some of the experiments in chapter 3. Dr. Arno Krotzky assisted with the planning of some of the experiments in chapter 4 and collaborated in the execution of experiment 11 of that chapter. Experiment 12a in chapter 5 was performed by Dr. Ben Bohlool and Ms. Patricia Nakao at the University of Hawaii in collaboration with me.

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ABSTRACT

This thesis focused on the host control of nodule initiation and development in the soybean (Glycine max. *[L.] Merr)* - Bradyrhizobium *symbiosis. In particular, the involvement of an internal or autoregulatory mechanism was investigated.*

It is proposed that in the wild-type cultivar Bragg, infection signals from the cortical cells of inoculated roots are translocated to the shoots, and result in the induction of a systemically translocatable, inhibitor substance, the concentration of which increases progressively after inoculation with Bradyrhizobium japonicum *strain USDA110 or exposure to high level nitrate.*

Conversely, nts382 is a nitrate tolerant, super-nodulating soybean mutant, with an altered autoregulation system, caused by a lowering or suppression of the inducible signal. This thesis provides evidence to suggest that the mutation occurs in the abscisic acid pathway, such that the mutant has a lower endogenous level of ABA, and is less sensitive to nodule suppression by ABA application than its wild-type parent cultivar.

Split root nodulation analysis using other nts mutants (nts1116 and nts1007) and wild type cultivars of soybeans (Williams and Clark) demonstrated a difference in their susceptibility to suppress nodulation and root weight partitioning, indicating that the autoregulation response is cultivar dependent.

Wild-type cv. Bragg and mutant nts382 were grown in split root systems to investigate the effect of timing of inoculation on nodule suppression and partitioning of root material to either side. For Bragg plants, grown in either the presence or absence of low level nitrate, nodulation of the second side was significantly suppressed when inoculation of the second side was delayed until 24 hours after the first side. Total nodule autoregulation of the second side was caused by a 7 day prior inoculation of the first side.

In the absence of nitrate the first inoculated side developed a larger root system than the second. It was originally thought that the carbohydrate deprivation hypothesis could be used to explain the minus nitrate data in that a limitation of structural resources could prevent nodulation and root growth of the second side. However, when nitrate was added to the nutrient solution of similar plants the root weight distribution was equalized but autoregulation still occurred. In other words, in the absence of nitrate, the clear demonstration that autoregulation is caused by a systemically translocated inhibitor substance is confused by a corresponding reduction in root weight partitioning on the delayed side, caused by nitrate limitation.

Conversely, nts382 grown in the presence of low level nitrate demonstrated no such nodule suppression. However, when nts382 plants were grown in the absence of KNO_3 as an external nitrogen source a 7 day delay in inoculation of the second side resulted in a 78 % suppression of nodulation. In both cases, nts382 plants developed equal size roots, regardless of the nodulation pattern. This indicates that nodule number is not a function of root size since autoregulation occurred in split root systems where the root weight on either side was the same. Further, it was shown, by means of ^{14}C - sucrose translocation studies, that autoregulation is not caused by a reduced translocation of photosynthate to the roots.

Attempts to suppress the nts phenotype by foliar and inter-cotyledonary injections of phyto-hormones were only partially successful since nodule number decrease was notable but did not decrease to wild type levels and patterns without affecting plant growth and development. However, daily injections of GA_4 (in the range 1 ng to 5 µg) significantly suppressed nodulation of nts382 plants while having no effect on Bragg nodulation. Additionally, nts382 was less susceptible to ABA induced nodule suppression than Bragg, by a factor of 50. This suggests that nts382 (being an autoregulation mutant) has a lowered gibberellic acid pool and hence requires a higher concentration of ABA to block the gibberellic acid pathway in order to suppress nodulation.

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CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION TO BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation is the process whereby atmospheric di-nitrogen (N_2), which constitutes 78 % of the air by volume, is reduced from its chemically inert form to ammonia (NH_3). By combining with hydrogen, nitrogen becomes "fixed," and so is able to enter biological systems.

Although certain free living prokaryotes are able to fix atmospheric nitrogen (e.g. *Klebsiella* and *Azotobacter* spp.) no higher organism alone has this capacity unless it enters into a symbiotic arrangement with a species of nitrogen fixing bacteria. Examples include the *Azolla - Anabena, Azospirillum - Paspalum* rhizosphere interactions and the *Frankia - Alnus* and legume - *Bradyrhizobium* or -*Rhizobium* (see Jordan 1982)[1] symbioses.

In the latter associations, specific soil bacteria of the genus *Rhizobium* or *Bradyrhizobium* enter into a symbiotic arrangement with a particular legume. The specialized environment which is formed as a result of this association is the root or stem nodule; inside which, the prokaryotic encoded enzyme nitrogenase converts N_2 to ammonium (Bergersen 1982). This fixed nitrogen is exported from the nodule in the form of amino acids, amides and / or ureides (Atkins 1984) for protein synthesis and general nitrogen metabolism in the host. In return, the plant provides an oxidizable carbon substrate (most likely succinate and malate, see Udvardi *et al.* 1988, Ronson and Astwood 1985) to the *Rhizobium* bacteroids as an energy source for metabolism and nitrogen fixation.

This thesis focuses on one of these symbioses: i.e. the one involving *Glycine max* (L.) Merr (soybean) and *Bradyrhizobium japonicum*. The initiation, development and functioning of nodules on the roots of soybean is regulated at three distinct levels.

The first level of regulation affecting the symbiotic process involves the basic genetic factors of both the host and the bacteria. The genes for nodule initiation, development and functioning, (the so called *nod* and *nif* genes) are found in both the host and prokaryote genomes (see Gresshoff and Delves 1986; Kondorosi *et al.* 1986 for an overview of both plant and bacterial genetics).

The analysis of the host's involvement is even possible at the molecular level. Host derived mRNA encodes for a class of proteins called "nodulins" which are found in

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[1]Because of the recent taxonomic separation of *Rhizobium* and *Bradyrhizobium* (Jordan , 1982), these terms will be used as referenced historically. As this thesis deals entirely with *Brady-rhizobium japonicum* strain USDA 110 or CB1809, the term *Brady-rhizobium* will be used exclusively to specify the here described symbiosis.

nodules and not in the supporting root (Legocki and Verma 1979; Nap *et al.* 1986). Examples of nodulins are leghaemoglobin, uricase, glutamine synthetase and sucrose synthetase. Bisseling *et al.* (1985) describes other nodulins which are expressed early in nodule development (such as ENOD2).

The second level of regulation involves the environmental influence of the soil ecology (see section 1.3). Physio-chemical and biological factors such as moisture, temperature, acidity, bacterial competition and mineral nutrition are all known to affect the extent of development of symbiotic nodulation (Dart 1974; Bohlool *et al.*1986; Hodgson and Stacey 1986). However, combined soil nitrogen is regarded as the primary factor restricting the extent of symbiotic development (see Beringer 1984; Carroll and Matthews 1988 for summary reviews). Indeed, biological nitrogen fixation is only a secondary mechanism for obtaining fixed nitrogen, as all stages of nodule formation and function may be suppressed while there is sufficient combined nitrogen available, usually in the form of soil nitrate (see Brill 1980). The phenomenon of nitrate inhibition of nodulation and nitrogen fixation is discussed in section 1.3.4.

The third level of regulation is mostly dependent on the host plant which mediates an internal or autoregulatory response in regard to the number, pattern and size of nodules which form on the roots (see section 1.4). This thesis primarily focuses on aspects of internal autoregulation.

1.2 GENETIC AND BIOCHEMICAL REGULATION OF LEGUME NODULATION

The establishment of a nitrogen fixing nodule is a stepwise process that can be arrested in a number of different stages which have been elucidated by studying plants inoculated with mutant bacteria. From these studies it is apparent that events which occur in the symbiosis consist of three major levels, namely: 1/ pre-infection, 2/ infection and nodule development and 3/ nodule function.

1.2.1 PRE-INFECTION EVENTS: COLONIZATION AND ATTACHMENT

The success of the association depends on genetic compatability between the plant and the *Rhizobium*. For example :

Bradyrhizobium japonicum and *Rhizobium fredii* infect and nodulate soybean (*Glycine max.*), and siratro (*Macroptilium atropurpureum*),
Rhizobium trifolii only infects and nodulates clover (*Trifolium repens*) and
Rhizobium meliloti only infects and nodulates alfalfa (*Medicago sativa*).

In fact, *Rhizobium* taxonomy is mostly determined by this inoculation group concept. Only recently have exceptions been noted. For example, many "cowpea" miscellany strains like NGR234 nodulate a wide range of legumes (Trinick and Galbraith, 1980).

Nodulation and host range genes are located on indigenous plasmids in *Rhizobium* species called *sym* plasmids (Hooykaas *et al.*1978; Johnston *et al.*1978; Rosenberg *et al.*1981; Banfalvi *et al.*1981). For example a 14 Kb Hind III fragment from the *sym* plasmid of *R. trifolii* strain ANU 843 has been shown to encode *nod* and *hsn* (host specificity genes), as transfer of this fragment to other *Rhizobium* sp. and *Agrobacterium tumefaciens* resulted in the aquisition of clover nodulation capacity (Schofield *et al.* 1984). In contrast, *Bradyrhizobium* strains do not possess *sym* plasmids and the equivilent *nod, hsn* and *nif* genes are located on the chromosome (see Hennecke *et al.* 1986).

1.2.1.1 Lectin hypothesis

Little is known about the nature and functioning of the recognition mechanisms despite many reviews on the subject (Albersheim and Anderson-Prouty 1975; Bauer and Bhuvaneswari 1979; Dazzo 1980). Until recently, it was widely postulated that host encoded lectins (sugar binding proteins) on the root surface interacted selectively with microbial cell surface carbohydrates, to act as determinants of host specificity (for review see Bauer 1981).

In effect, the lectin is depicted as a specific glue holding the bacteria to the root, allowing nodule initiation and development to follow. Bauer (1981) suggested that previous recognition models (e.g. Dazzo and Hubbell 1975; Bohlool and Schmidt 1978) failed because they did not indicate any functional difference in terms of infection and nodulation between (clover) lectin mediated attachment and attachment that was not mediated by (clover) lectin. He proposed another model reflecting evidence from Albersheim and Anderson-Prouty (1975), Bauer and Bhuvaneswari (1979) and Graham (1980).

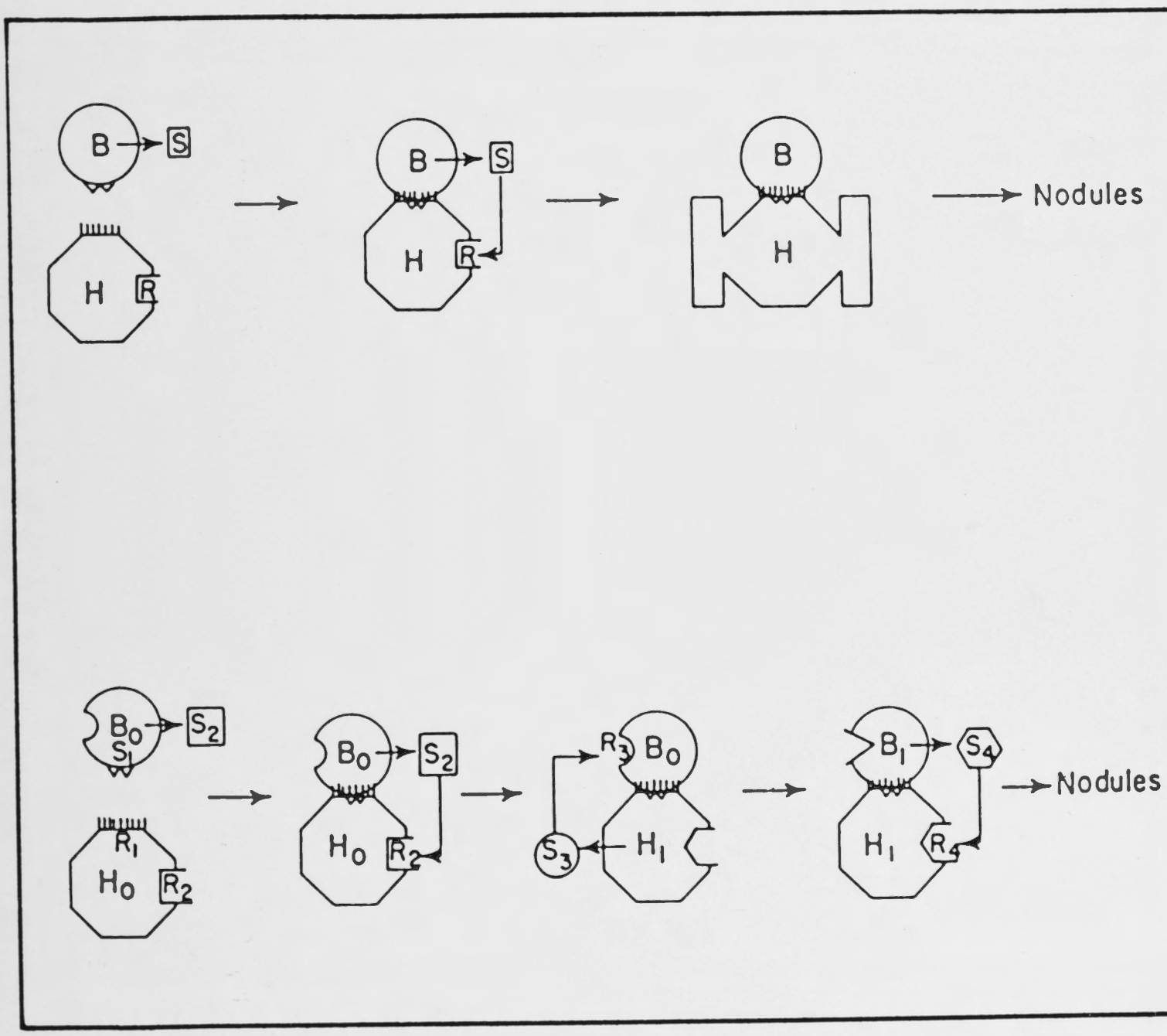
In a simple model, Bauer has the *Rhizobium* attaching to a suitable host cell by some unspecified mechanism. An extracellular substance or surface component of the *Rhizobium* interacts with a receptor substance on the surface of the host cell. The interaction is coupled so that it triggers certain responses in the host that lead to its infection and nodulation (see figure 1.1). As in the previous model, the "receptor" could be a host lectin and the signal substance could be a cell surface polysaccharide.

Figure 1.1 Model 1 of plant - bacterial recognition (after Bauer 1981)

B = bacteria, H = host, R = receptor, S = signal

Figure 1.2 Model 2 of plant - bacterial recognition (after Bauer 1981)

B_0 = Bacteria, H_0 = host, S_1 = signal 1, R_2 = receptor 2,
S_2 = signal 2, R_2 = receptor 2, S_3 = signal 3, R_3 = receptor 3,
S_4 = signal 4, R_4 = receptor 4


B
S
H
R
Nodules
B0
S1
S2
R1
H0
R2
R3
S3
H1
B1
S4
R4

A more complex model has been suggested by Bauer (1981) since recognition is probably not a single step process. The model involves a series of signal production, signal reception, and reception induced responses between the symbionts (see figure 1.2).

However, the lectin binding hypothesis has not been firmly substantiated, and may only be part of the wide range of processes which determines host range. Indeed, Robertson *et al.* (1981), have shown that *Rhizobium* strains can bind to hosts that they do not nodulate. Further, Vesper and Bauer (1984) have implicated the pili produced by *B. japonicum* as being the primary mediators of attachment since antiserum produced against the pili inhibited *Rhizobium* attachment and subsequent nodulation.

Halverson and Stacey (1984, 1985) were able to correct a *Rhizobium* strain, with a slow-to-nodulate phenotype, by incubating the bacteria in soybean root exudate before inoculation onto the host. This suggests that soybean lectin is not the direct mediator of the *B. japonicum* attachment to soybean roots but may have a role, perhaps in conditioning the *Rhizobium* for nodulation. Alternatively, it is possible that HS111 gets corrected by isoflavones contained in the root exudate which contain low level lectin properties. This is a possibility since no tests for purity were run. This lends support to Bauer's (1981) second model (see figure 1.2), that is, the receptor - signal - response cycle, which could also be used to support the flavone / isoflavone theory.

1.2.1.2 Induction of *Rhizobium nod* genes by flavones / isoflavones

In *Rhizobium* the *nod* genes are located on *sym* plasmids and are organized in several transcriptional units, namely, *nod* ABC, *nod* D, and *nod* FE (Downie *et al.* 1985, Rossen *et al.* 1984) while the nod genes of *Bradyrhizobium* are chromosomally located. In both cases only one gene, namely *nod* D, is expressed in culture. *Nod* D is thought to be a fast-acting regulatory gene, whose product interacts with inducer molecules from the legume root exudate to activate the transcription of other bacterial *nod* genes via nod box activation (Rossen *et al.* 1985).

Kosslak *et al.* (1987) identified the isoflavones daidzein and genistein, (which induce *nod* C -lac Z fusions of *B. japonicum* strain USDA123), as the major components in soybean root extract responsible for inducing the *nod* genes in *B. japonicum.* However, in *R. meliloti, R. trifolii and R. leguminosarum,* flavones (Peters *et al.* 1986, Redmond *et al.* 1986) and flavonones (Firmin *et al.* 1986) have been identified as the components of legume extracts which induce the *nod* genes. Interestingly, Firmin *et al.* (1986) have identified daidzein and genistein as strong antagonists of *nod* gene induction in *R. leguminosarum.*

Thus, bacteria in the rhizosphere are primed for the presence of a plant through these exudates. Appropriate bacterial genes, some of which may function in flavone / isoflavone breakdown, are induced, thus permitting the further development of the infection process.

Isoflavonoids, as a class, include substances associated with defence mechanisms against microbial pathogens. Phyto-alexins (from the Greek *phyton* meaning plant; *alexin* meaning protecting substance) accumulate rapidly in plant-pathogen interactions and possess anti-fungal and anti-bacterial properties. Over 100 phyto-alexins have been isolated from many (mostly dicotyledenous) plant species. The predominant phytoalexin in the family *Leguminosae* are the iso-flavonoids (Ersek and Kiraly 1986). These compounds are not usually present in plant tissues prior to infection. Infection is required to induce their synthesis. The substances which induce the plants to synthesize phyto-alexins are referred to as elicitors and are usually of carbohydrate, protein or lipid origin.

Therefore the involvement of plant exudates such as isoflavones may assist in the explanation of some host-range / recognition observations. Indeed a plant exudate - *nod* D induction of *Bradyrhizobium* common nod genes fits Bauer's (1981) model.

1.2.2 INFECTION EVENTS: ROOT HAIR CURLING

The infection of leguminous plants may proceed via penetration of the root hair by rhizobia. This apparently occurs through a process of the invagination of the root hair cell wall, (with subsequent infection thread formation as seen in clover, soybean and siratro), or via a direct method in which rhizobia invade the host cell cytoplasm through fragile points in the cell wall, in which subsequent infection thread formation may or may not occur (see section 1.2.6 - Alternative modes of legume infection).

The following section will deal with infections initiated via root hair curling and infection thread formation in soybean (*Glycine max* (L.) Merr) (see figure 1.3 from Gresshoff and Delves 1986), Reference will be made to the similar, but not identical clover infection process (see Vincent 1980).

Soybean root hair infection always occurs in the markedly curled region of the root hair (Turgeon and Bauer 1982, 1984). Mature root hairs are generally not receptive to *B. japonicum* invasion (Calvert *et al.* 1984) and nodules usually are initiated in the region of the root hair that is closest to the root tip at the time of inoculation (Bauer 1981). Root hair cells develop from root epidermal cells called trichoblasts and it is these cells to which *B. japonicum* attach preferentially. This attachment appears to initiate sub-epidermal cell divisions which can be observed prior to infection thread formation (Calvert *et al.* 1984;

Figure 1.3 **Model of nodule ontogeny in the soybean - *Bradyrhizobium* symbiosis**
(from Gresshoff and Delves 1986)

V = vascular bundle, **P** = pericycle (the site of the lateral root formation),

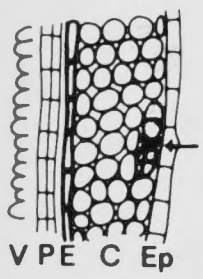
E = endodermis, **C** = cortex, **Ep** = epidermis, **PC** = plant cortex,

LC = lenticel, **SC** = scleroid cell, **IZ** = infected zone,

NOC = nodule outer cortex, **NIC** = nodule inner cortex,

PDT = peicycle derived tissue, **BCL** = boundary cell layer.

Stage one:



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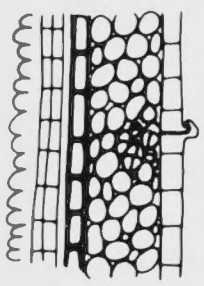

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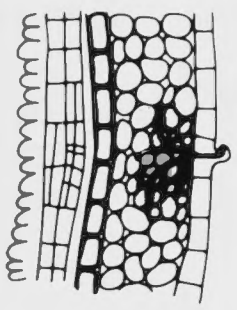

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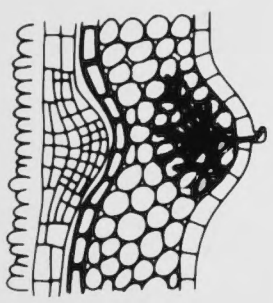

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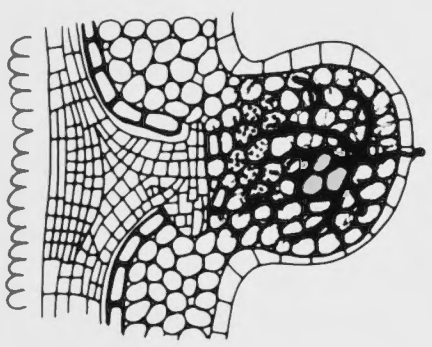

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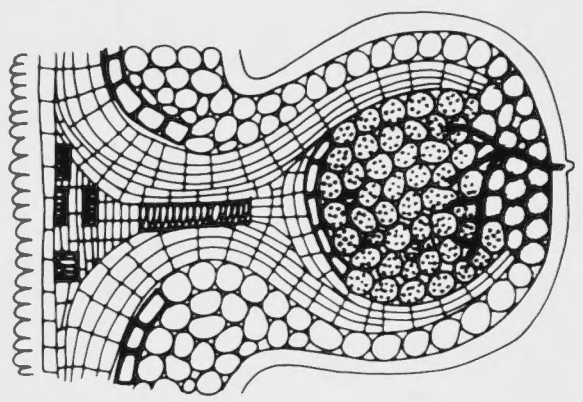
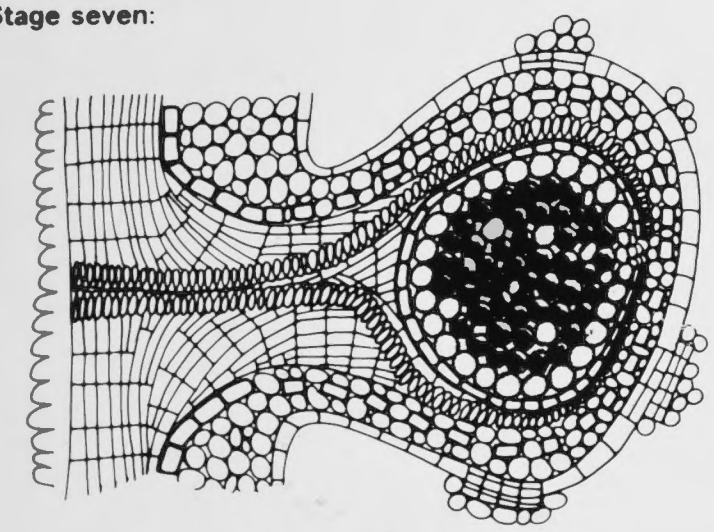
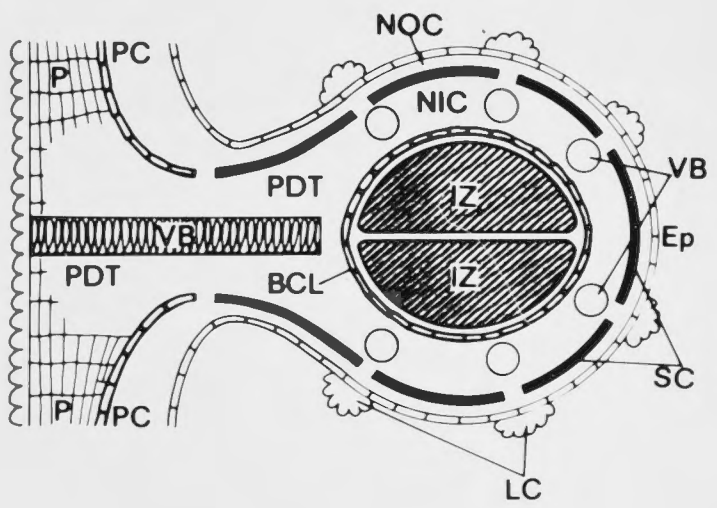


Stage seven:



Stage eight: schematic—side on



Proposed scheme of soybean nodule development

see also stage 1, Figure 1.3). Recent evidence by Bauer *et al.*(1985) even shows sub-cortical cell divisions (pseudo-infections) can even be induced by trans-membrane located *Rhizobium* cells.

The attachment of the *Rhizobium* to the trichoblast also may cause the curling of that hair. Bauer (1981) has proposed a model of root hair curling induction by rhizobia (see figure 1.4).

In this model, (see figure 1.4a) a hair is emerging from the apical end of a root epidermal cell. The flexible ***a*** layer tip of the hair bulges outwards as the result of turgor pressure against an area of the localized removal of the ***β*** layer (which is presumed to be a consequence of host induced disintegration of the micro-fibrillar matrix or inhibition of the ***β*** layer synthesis).

It is also presumed that deposition of new ***a*** layer material is heaviest at the apex to the edge of the hemisphere swelling. As a result, any *Rhizobium* cell attached to the emerging tip will gradually be displaced from the apex to the edge of the hemisphere (see figure 1.4b).

The model proposes that curling results from a localized inhibition of the ***β*** layer deposition in the emerging hair, induced by an attached *Rhizobium* cell. Thus the rigid cylinder of ***β*** material thus does not develop past the attached *Rhizobium* cell but continues to be deposited inside the ***a*** layer opposite the attached *Rhizobium* (see figure 1.4c). As the hair elongates, the flexible hemispherical tip gradually pivots around, resulting in a tight curl that envelops the *Rhizobium* (see figure 1.4 d and e).

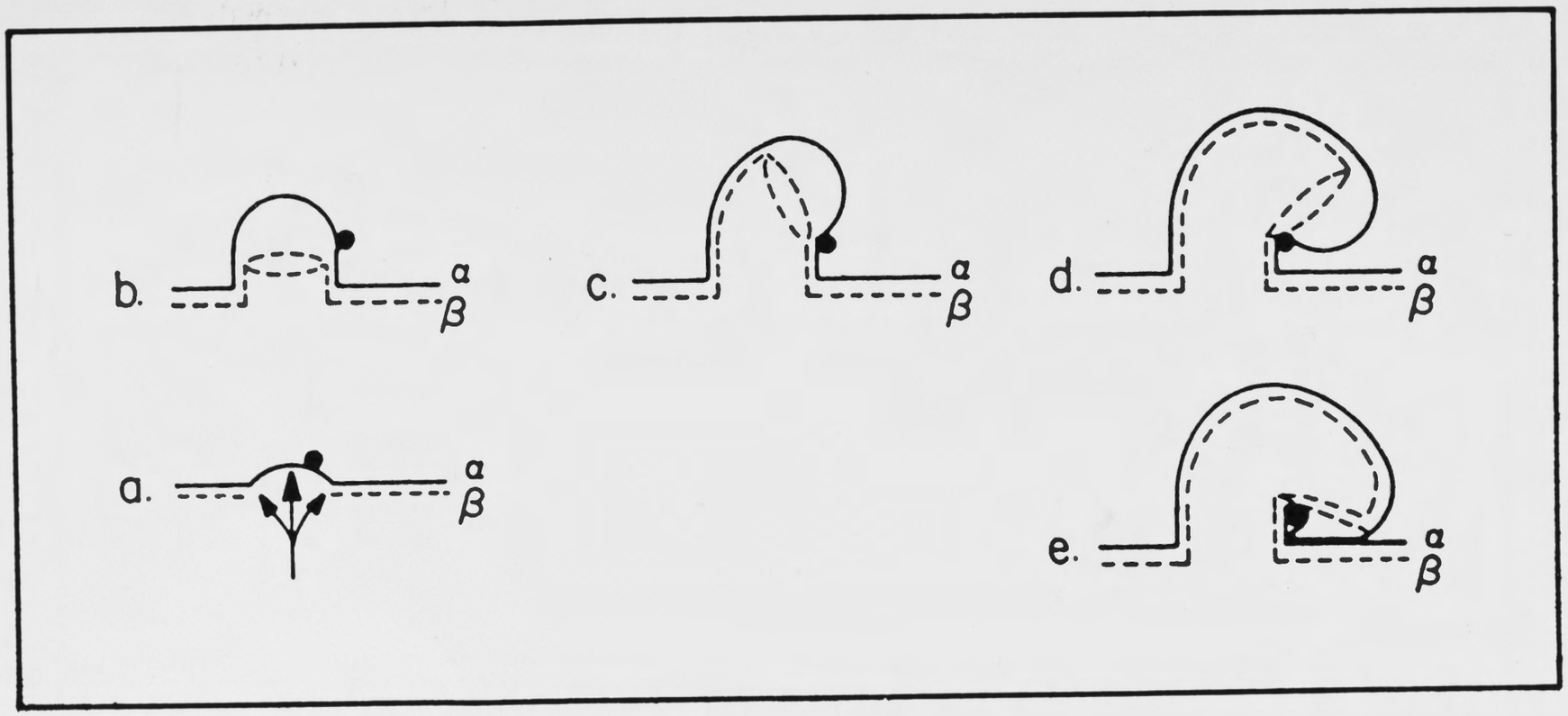
1.2.3 INFECTION THREAD FORMATION

The soybean infection thread is initiated directly below the position of the bacterial colony in the pocket of the curled root hair. Infection threads are tubular structures that carry the *Bradyrhizobium* cells, often single file, from the root surface into the root cortex (Bauer 1981). After penetration by the infection thread, cortical cells divide in advance of the ramifying thread (see stage 2 figure 1.3). Such hypodermal cell divisions may occur without infection and are called "pseudo-infections". This suggests the action of a diffusible bacterial-derived factor which induces these cell divisions in the appropriate cortical tissue. Matthews (1987) found that soybean often have multiple infection threads within the one root hair cell.

Figure 1.4 **Model of root hair curling induction by rhizobia** (after Bauer 1981)

α = alpha layer, β = beta layer,

• = bacteria


b.
α
β
c.
α
β
d.
α
β
a.
α
β
e.
α
β

1.2.4 NODULE DEVELOPMENT

Increased cortical cell activity "signals" the pericycle tissue to initiate divisions (see figure 1.3, stage 3), which results in a visible lump. Further pericycle divisions distort and eventually rupture the endodermis (see figure 1.3, stage 4). The rhizobia are eventually released from their infection threads into the cortical cells where they begin the transformation to the bacteroid or nitrogen fixing state. At the same time, pericycle tissues push through the endodermis and proliferate towards the invaded (shaded) zone (figure 1.3, stage 5).

Vascular traces develop around the infected zone in the nodule via a development of the pericycle, and possibly the cortical cells. The tracheids at first develop in parallel to the root axis, often in unconnected cell clusters, but then grow together to form vascular strands perpendicular to the root axis (indicated by spiral tissue). The early boundary cell layer around the infected zone is noticeable, as is the formation of an endodermis like layer in the nodule cortex (see figure 1.3, stage 6 and also figure 1.5).

The fully developed nodule is characterized by the following properties: the bacteroids in the infected zone are actively converting di-nitrogen to ammonium via the action of the nitrogenase enzyme complex; the infected zone consists of infected and uninfected cells; vascular bundles are connected and surround the infected tissue; scleroid (thick walled) cells develop in a nearly continuous layer in the nodule cortex, with the gaps being associated with the position of vascular bundles and the location of lenticels. (figure 1.3, stage 7). Stage 8 of figure 1.3 indicates some possible cell lineages in the fully developed nodule.

1.2.5 NODULE FUNCTIONING

1.2.5.1 Nitrogenase activity and nitrogen assimilation

Within the fully developed nodule, a favourable environment exists for the bacteroid contained nitrogenase enzyme to reduce atmospheric nitrogen to ammonium . The overall reaction is :

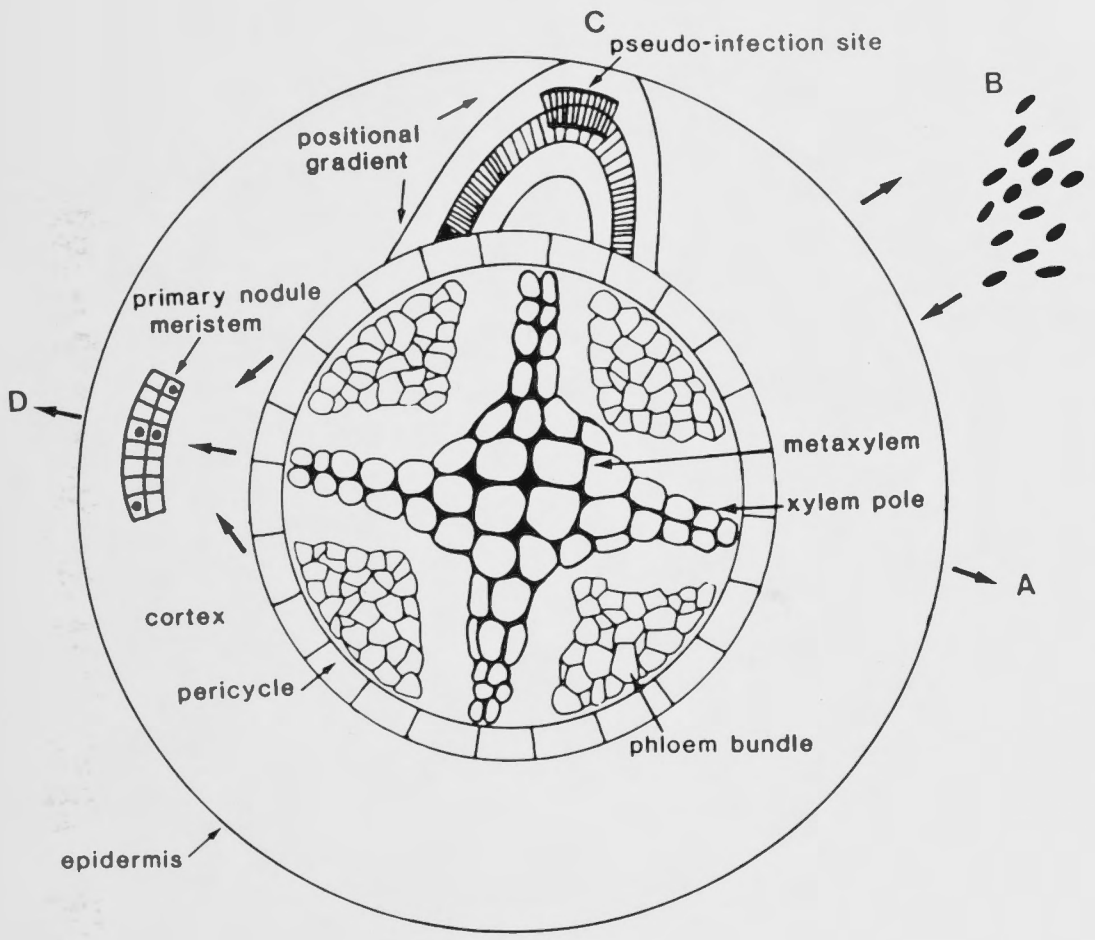
$$N_2 + 10H^+ + 16ATP + 8E^- \xrightarrow{\text{NITROGENASE}} 2NH_4^+ + H_2 + 16ADP + 16Pi$$

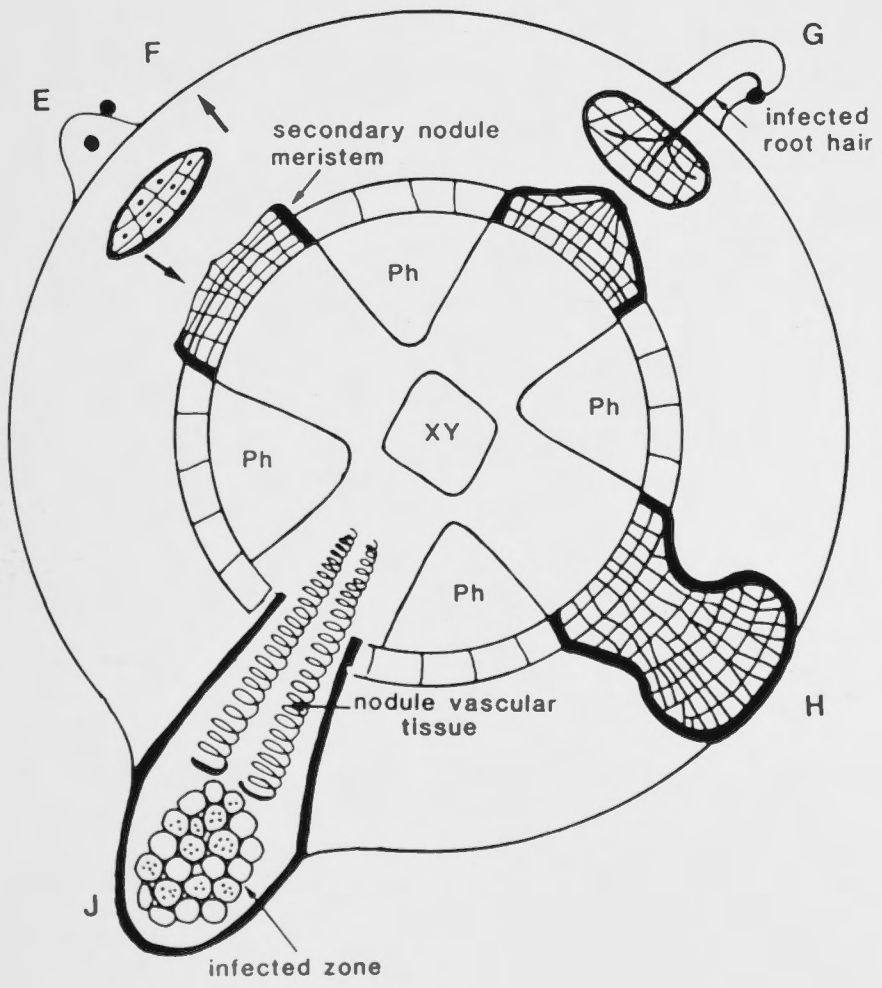
The ammonium produced in this reaction is initially incorporated into glutamine via the enzyme glutamine synthetase (GS) :

Figure 1.5 **Model of soybean nodule initiation**
(from Rolfe and Gresshoff 1988)

Where: **Xy** = xylem, **Ph** = phloem,
A = Root derived stimulator,
B = Bacterial cell derived division factor,
C = Plant cell derived division factor (ex phloem),
D = As for C, but ex meta xylem,
E = Signal from infected cortical cells to secondary nodule meristem,
F = Signal from secondary nodule meristem,
G = Infection thread formation,
H = Proliferation of cortical cell divisions,
I = Formation of nodule vascular tissue,
J = Nodule formation.

SOYBEAN NODULE INITIATION





$$\text{GLUTAMATE} + \text{ATP} + NH_4^+ \xrightarrow{\text{GLUTAMINE SYNTHETASE}} \text{GLUTAMINE} + \text{ADP} + \text{PI}$$

This product is transaminated to yield glutamate via the enzyme glutamine oxoglutarate amino-transferase (GOGAT) :

$$\text{GLUTAMINE} + 2\,\text{OXOGLUTARATE} + \text{NADPH} + H^+ \xrightarrow{\text{GOGAT}} 2\,\text{GLUTAMATE} + NADP^+$$

The glutamate is transaminated further or is recycled as a substrate for the glutamine synthetase reaction (see Boland *et al.* 1980). Following the incorporation of newly fixed nitrogen into glutamine via the GS-GOGAT pathway in the nodule cytosol, nitrogen must be excreted into the xylem and transported to other parts of the plant where it is utilized. In soybean, the major nitrogenous compound transported from the nodules are the ureides allantoin and allantoic acid (see Matsumoto *et al.* 1977; and Schubert and Boland 1984). Their synthesis proceeds via an involvement with uninfected cells in the nodule.

1.2.5.2 Carbon metabolism in the nodule

In order for the energy expensive process of di-nitrogen reduction to occur, the nodules must be supplied with an adequate amount of photosynthate (see Reibach and Streeter 1983), which is translocated to the nodules in the form of sucrose (Bach *et al.* 1958). In elementary terms, sucrose (in soybean nodules), is initially degraded via the enzymes alkaline invertase and sucrose synthetase (Morrell and Copeland 1984, 1985) to yield fructose and glucose which are further phosphorylated by fructokinase and hexokinase respectively.

It is thought that further metabolism occurs via glycolysis and the TCA cycle. The carbon compounds made available to the bacteroid by the legume cell are gradually being elucidated. Experiments with catabolism mutants and uptake studies have suggested a specific role for succinate, fumarate or malate in fuelling nitrogen fixation (Dilworth and Glenn 1984; Ronson and Astwood 1985; Price *et al.*1987; Udvardi *et al.* 1988).

Two of the major by-products of bacteroid nitrogen fixation are H_2 and CO_2 (Bergersen 1982). The energy loss by H_2 evolution accounts for about 25 % of the total amount used by nitrogenase (Brewin 1984), although some *Rhizobium* strains possess a hydrogen uptake system (encoded for by *hup* genes) which can reduce this loss (Brewin 1984) by a regeneration of ATP and H_2O. However, this process may have limited symbiotic significance as O_2 is a necessary substrate and may be quite limiting within the

nodule interior (see next section). *Hup* genes may be more important for free-living organisms.

The CO_2 which is respired as a result of the nitrogen fixation process can be metabolized in the nodule to yield oxaloacetate via the enzyme PEP carboxylase. This fixed carbon can then be used in the synthesis of (1) carbon skeletons for the assimilation of fixed nitrogen, (2) respiratory substrates for the bacteroids and (3) carboxylic acids to balance excess cation exchange (See Gadal 1983, Schuller *et al.*1986).

1.2.5.3 Oxygen supply in the nodule

A large flux of oxygen is required by both the bacteroids and the host cytoplasm for oxidative phosphorylation (Bergersen and Turner, 1967), however the enzyme nitrogenase is extremely sensitive to oxygen inactivation (Bergersen 1974). Oxygen diffusion into the nodule is firstly regulated by the physical barrier of the nodule cortex, with gas exchange being via the lenticels (Sinclair and Goudriaan 1981).

Once inside the nodule, it has been suggested that oxygen supply may also be regulated by a variable diffusion barrier for many legume nodule types (Minchin *et al* 1983, Sheehy *et al.* 1983). Carroll *et al.* (1987) also demonstrated this barrier for soybean nodules. Further, Sinclair and Gourdriaan (1981) postulated that this diffusion barrier would not substantially inhibit the entry of N_2 into the nodule. Recent studies by Carroll *et al.* (1987) showed that both dark and nitrate induced loss of nitrogenase activity can be partially resumed by the addition of extra oxygen, suggesting that such senescence phenomena also involve the barrier.

Finally, an iron containing leghaemoglobin protein acts to regulate the supply of oxygen to the bacteroids (Broughton and Dilworth 1971; Appleby 1984). Bacteroids of most legumes scavenge about 1-10 μM free oxygen through the action of leghaemoglobin specific oxidases which accept oxygen from the haemoglobin molecule (Gresshoff and Delves 1986). The haem molecule is thought to be a product of the bacteria and is excreted into the plant cytoplasm during bacteroid development, whilst the globin moiety is a product of plant genes (Appleby 1985). Leghaemoglobin appears to be localized in the cytoplasm and not the peri-bacteroid space of pea, lupin (Robertson *et al.* 1984) and soybean (Verma *et al.* 1985) nodules. However, the method of transport across the peri-bacteroid membrane remains unclear.

1.2.6 ALTERNATIVE MODES OF LEGUME INFECTION

Although *Rhizobium* infection via root hair cells has been demonstrated to be the mode of entry in several temperate crop legumes such as pea, bean, clover, alfalfa and soybean, a variety of alternative infection mechanisms have been observed in other legumes (see Rolfe and Gresshoff, 1988).

For example, in peanut (*Arachis hypogaea*) and *Stylosanthes* spp., infections may proceed without the involvement of root hair infections. Here, infections may be initiated at the basal junctions of lateral roots and adjacent epidermal cells. The rhizobia progress inter-cellularly via the alteration or disintegration of the host cell wall, and are subsequently enclosed in membrane envelopes (see also Chandler 1978; Chandler *et al.* 1982. It has been suggested that infection through a gap in the host epidermis would remove part of the specificity due to chemical binding (Dazzo and Hubbell 1975).

Apart from forming nodules on their roots, some legumes have the capacity to develop stem nodules. This phenomenon has been observed in only three known genera and of these, the best studied have been *Sesbania rostrata* (Dreyfus and Dommergues 1981; Duhoux and Dreyfus 1982; Rinaudo *et al.* 1983, Tsien *et al.* 1983, Duhoux 1984; Olsson and Rolfe 1985); *Aeschynomenae indica* (Arora 1954; Yatazawa and Yoshida 1979; Yatazawa and Susilo 1980; Stowers and Eaglesham 1983; Legocki *et al* 1983) and *Neptunia oleracea* (Schaede 1940).

Thus, stem nodulation conveys the advantage in that *Rhizobium* are able to infect an aerial site which (a) remains infectable for many months or until the dormancy of the adventitious root is broken, (b) is relatively free from the competitors which abound in the soil and often colonize the infection sites, (c) is less affected by the high soil nitrate concentrations which can inhibit root nodulation and (d) is not affected by waterlogged soil conditions which normally result in oxygen deprivation.

1.3 ENVIRONMENTAL FACTORS REGULATING NODULE FORMATION

The success of the legume symbiosis also depends on environmental influences of the ecosystem. Physio-chemical and biological factors such as moisture, acidity, salinity, and mineral nutrition are known to affect the extent of nodulation. While a brief description of these factors will be included, it must be noted that the primary environmental factor restricting the extent of nodulation is generally the concentration of exogenous nitrate in the soil. Symbiotically active legumes are able to utilize either soil nitrate (combined nitrogen) or atmospheric di-nitrogen as a nitrogen source (e.g. see

Carroll and Gresshoff 1983). Soil nitrate is used in preference (Carroll *et al.* 1985a; Becana and Sprent 1987), and so, either the initiation, development or functioning of the nodules is suppressed. Thus, the normal legume crop removes nitrate from the soil despite its natural ability to fix high amounts of nitrogen from the atmosphere (Herridge *et al.* 1984). A more detailed analysis of nitrate inhibition of nodulation of the soybean symbiosis is in section 1.3.4).

1.3.1 WATER STRESS

Both drought and water-logging can strongly inhibit nodule development and acetylene reduction, and may also reduce nodule longevity. Using a detached nodule system of soybean, it was shown that as nodules dried out there was a steady loss of acetylene reduction once the water loss exceeded 20 % of the initial nodule fresh weight (Sprent 1971). It was also shown that there was a collapse of cytoplasmic structure in the vacuolated cells of the nodule cortex (Sprent 1972) and splitting of cell walls and rupturing of plasmodesmata connections in the bacteroid containing zone (Sprent 1971). It was suggested that cortical cell collapse inhibited nodule activity by reducing the diffusion of oxygen into the bacteroid containing zone (Pankhurst and Sprent 1975).

Water-logging is usually considered equally damaging to fixation (Huang *et al.* 1975) with appreciable rotting and detachment of nodules occuring after only four days of inundation. Water-logging probably acts by reducing the underground supply of oxygen. Low pO_2 inhibits the growth of nodulated plants (Ferguson and Bond 1954) but the effects on nodule longevity may not be so marked (Criswell *et al.* 1976). Such water stresses must now be looked at in view of the variable O_2 barrier as reported by Sheehy *et al.* (1983); Minchin *et al.* (1985) and Carroll *et al.* (1987). If there is a variable barrier, then the effects noted by Sprent may be too severe and perhaps are an artifact of the isolated (detached) nodule system.

1.3.2 SOIL PH AND SALINITY

Legume species differ in their susceptibility to soil acidity. Studies have shown reduced growth, nodulation and yield in plants grown in soils with a pH less than 5.0 (Munns 1977 a,b). The reasons for this have been variously associated with the hydrogen ion concentration (Andrews 1978), the high percentage saturation of aluminium and manganese in the soil solution (Andrews 1978), and to calcium and molybdenum deficiencies (Franco 1978; Graham and Chatel 1983). The interaction between different acid soil factors on nodulation and nitrogen fixation has been reviewed extensively (Munns 1977 a,b; Andrews 1978; Robson 1983) and will not be detailed here.

External factors like pH or Al may interact with the internal nodulation control system and generate the reduced symbiotic phenotypes. For example, at alkaline pH symbiotic function is impaired as it induces deficiencies of manganese, zinc, boron and iron (Hallsworth 1972; Lindsay 1978); and soil salinity (Singleton 1983). Conlan (1987), looked at the plant and bacterial contributions to pH sensitivity in the soybean - *Bradyrhizobium* symbiosis and found that complete inhibition of genotypes Bragg and nts382 nodulation at pH 4.0 was caused by the localised effects of acidity on the host root, since *B. japonicum* infectivity was not sufficiently reduced to completely prevent nodulation at pH 4.0.

1.3.3 MINERAL NUTRITION

The supply of available mineral nutrients in the soil may affect the growth and persistence of rhizobia, infection, nodule development and nodule function as well as affecting host plant growth (Robson 1983). There has been little work done on the negative external requirements for growth of *Rhizobium* in the soil. However, qualitative requirements for growth have been demonstrated for P, K, Mg, Ca, Mo, Co, Zn, Mn and Fe (Robson 1983). Many of these same nutrients are required for nodulation, however several nutrients are required in higher concentrations for these processes than for the host legume growth alone (Robson 1983). Such nutrients include Ca, Cu, Mo, Co, P.

1.3.4 REGULATION OF THE SOYBEAN SYMBIOSIS BY NITRATE

The soybean can readily use either symbiotic of combined nitrogen (see figure 1.6, also, Norman 1944; Thornton 1946; Allos and Bartholemew 1959; Harper 1974) but the amount of (nodulation and) symbiotic nitrogen fixation is inversely related to the amount of combined nitrogen available (Hinson 1975). Indeed nitrogen fixing activity is generally confined to geographical areas with low nitrate availability (Becana and Sprent 1987), since more energy is required to fix N_2 than to utilize NO_3^- (Sprent and Raven 1985).

The first step in the assimilation of nitrate in higher plants is the reduction to nitrite, which is catalysed by nitrate reductase:

$$NO_3^- + NAD(P)H + H^+ \xrightarrow{\text{NITRATE REDUCTASE}} NO_2^- + NAD(P^+) + H_2O$$

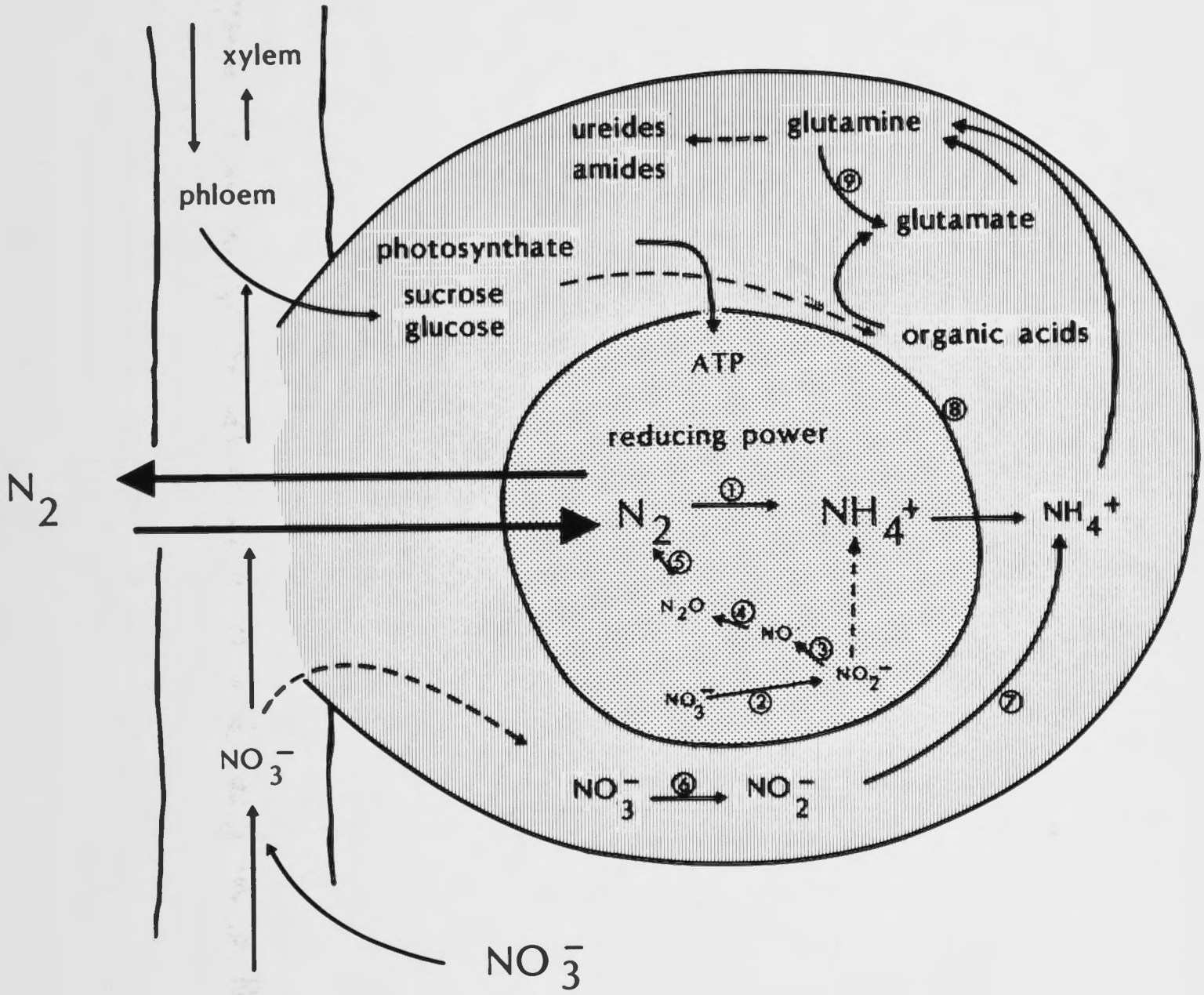
Nitrate reduction occurs mainly in the shoot in most species. Soybean has several nitrate reductases (Mortimer 1983), 2 constitutive and one inducible (Harper 1974; Spreit

Figure 1.6: Pathways of nitrate metabolism, representing the interactions among N_2 fixation, NO_3^- and NO_2^- reduction and NH_4 assimilation in legume root nodulation
(Re-drawn from Sprent and Becana 1987)

The dependence of these processes on photosynthate supply is indicated. The main carbon substrates utilized by bacteroids include sucrose, glucose and organic acids (e.g. succinate and malate). Dotted lines indicate steps of minor importance: NO_3^- incorporation into the nodules via connections with the root xylem, and assimilatory NO_2^- reduction in the bacteroids.

Enzymes in bacteroids: (1) nitrogense, (2) NR, (3) NiR, (4) NO-reductase.

Enzymes in the nodule plant fraction: (6) NR, (7) NiR, (8) glutamine synthetase, (9) glutamate synthase.


xylem
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photosynthate
sucrose
glucose
organic acids
ATP
reducing power
N_2
NH_4^+
N_2O
NO
NO_2^-
NO_3^-
NO_3^-
NO_2^-
NO_3^-
N_2
NH_4^+

et al. 1985 Carroll and Gresshoff, 1986). This is followed by the nitrite reductase catalysed reduction of nitrite to ammonium (see Pate and Atkins 1983):

$$NO_2^- \underset{RED}{+ 6\ FD} + 8\ H^+ \xrightarrow{\text{NITRITE REDUCTASE}} NH_4^+ + \underset{OX.}{6\ FD} + 2\ H_2O$$

While it is common knowledge that combined nitrogen (primarily as soil nitrate) has a general inhibitory effect on nodulation and nitrogen fixation, the actual mechanism(s) involved are still not clear (see Malik *et al.*1987; Carroll and Matthews 1988 for reviews). Inhibition seems to occur at three levels outlined below :

1.3.4.1 Effect of combined nitrogen on recognition and nodule initiation

Malik *et al.* (1987) have stated that nitrate inhibition produces evidence for a host regulatory mechanism that controls the establishment and functioning of the symbiotic association in relation to the supply of fixed nitrogen in the soil. The addition of nitrate to the roots of legumes has been reported to affect the symbiosis at a variety of stages, however the data still remains somewhat contradictory (Hinson 1975; Noel *et al.* 1982; Truchet *et al.* 1982; Carroll and Gresshoff 1983; Ralston and Imsande 1983; Gibson and Harper, 1985).

Further, Malik *et al.* (1987) indicate that for soybeans, (which were grown in plastic growth pouches), exposure to 15 mM KNO_3 inhibited tap root nodulation primarily at the level of initiation and infection. They showed that nitrate has its greatest effect on infection events which were completed within 18 hours of inoculation by *B. japonicum.* Consistent with other findings, they demonstrated that initial cortical cell division and infection thread formation were reduced by exposure to nitrate. However, they suggested that neither of these processes is complete within 18 hours of inoculation and that infection thread formation cannot be observed until approximately 24 hours after inoculation. The fact that cortical cells continued to divide throughout the infection development suggested that cortical cell divisions and infection thread formation may themselves be nitrate insensitive processes, with their apparent nitrate inhibition being dependent on other earlier events that are nitrate sensitive (and that are completed within 18 hours of inoculation).

Notwithstanding the above, the steps in nodule initiation and infection directly affected by nitrate may still include any of the following: (1) contact with bacterial and root exudates, as nitrate has been postulated to inhibit the accumulation of lectins in root cell walls and so block the recognition between the legume and the *Rhizobium* (Dazzo and

Gardiol 1984); (2) changes in the chemical composition of the root cell wall in response to nitrate treatment (Dazzo and Gardiol 1984) which suggests that nitrate may have a phyto-hormonal effect (Trewavas 1981); (3) suppression of root hair emergence (Thornton 1936; Munns 1968 a,b); (4) induced root hair curling and deformation (Thornton 1936; Darbyshire 1966; Munns 1968 a,b) and (5) initial induction of cortical cell division .

It has been suggested that indole acetic acid (IAA) is involved in the nodulation process since IAA alleviated some of the effects of nitrate on curling, infection thread formation and nodulation of *M. sativa* and reduced infection thread abortion (see Kefford *et al.* 1960; Tanner and Anderson 1963a; Valera and Alexander 1965).

It has been suggested further, that the inhibitory action of nitrate on root hair curling and infection thread formation could be based upon the catalytic destruction of IAA by nitrite (Tanner and Anderson 1963a). This view was based upon the following observations:

(a) IAA produced by rhizobia from tryptophan in root extracts is probably involved in root hair infection (Libbenga and Torrey 1973),
(b) "the major growth promoter of all nodule ages was IAA" , with extractable auxin levels per gram fresh weight of nodules being 40 - 60 times the level in the roots (Pate 1958),
(c) most rhizobia can reduce nitrate to nitrite (Tanner and Anderson 1963a) and
(d) nitrite catalytically destroyed IAA (Tanner and Anderson 1963a) .

However, Quiggin *et al.* (1988), provided evidence that nitrate, but not nitrite, has its inhibitory effect on nodulation via its accumulation inside the plant. They showed that the nitrate reductase soybean mutant, (NR328), inoculated with *B.japonicum strain* USDA110, and grown on 3 mM KNO_3, had only 70 % of the inducible nitrate reductase level of wild-type soybeans, and accumulated nitrate in its leaves. Thus if one is to accept the view that nitrite is involved in nodule inhibition, an increase in nodule mass on NR328 plants would be expected. However, this was not shown, with nodule mass being 3.2 ± 2.5 mg/g plant dry wt. compared to 14.6 ± 5.3 mg/g plant dry wt. on Bragg plants (D. Quiggin, pers. comm., see also figure 1.6).

1.3.4.2 Effect of nitrate on nodule development and functioning

Malik *et al.* (1987) reported that the addition of nitrate seems to affect nodule function both at the level of rapid reversible effects on nitrogen fixation and at the level of longer term, irreversible break-down of tissue organization.

Nitrate has been shown to inhibit established nitrogenase activity in nodules (Copeland and Pate 1970, Wong 1977, Houwaard 1980), lower leghaemoglobin levels, limit the development of nodule mass, and initiate senescence of nodule function (Chen and Phillips 1977).

Two possible mechanisms have been proposed to explain nitrate induced repression of nitrogenase activity by rhizobia. Firstly, it has been shown that nitrite is a potent inhibitor of nitrogenase activity in bacteroids extracted from nodules (Rigaud *et al.* 1973) and, secondly, it has been suggested by Virtanen (1950) that nitrite forms a NO - compound with leghaemoglobin, thus destroying its function as an O_2 supplier to bacteroids.

Studies by Gibson and Pagan (1977), using a nitrate reductase deficient mutant of the cowpea *Rhizobium* 32H1, have shown that neither of these forms of inhibition are responsible for the lowered levels of nitrogenase activity in nitrate treated plants, indicating that lowered nitrogenase activity in the bacteroids of plants supplied with nitrate is due to factors other than nitrite inhibition. It is nevertheless possible that nitrite formed in the nodule cytoplasm via the plant derived nitrate reductase may still be involved. Lawson (1986) looked at this situation in soybean using a NR- soybean plant plus a NR- *Bradyrhizobium*. His conclusions were that neither nitrate concentration inside the tissue, nor the products of nitrate reduction (or NR activity) are related to the degree of nitrate inhibition of nodulation.

An alternative explanation for the inhibition of nitrogen fixation has become known as the carbohydrate deprivation hypothesis (see Oghoghorie and Pate 1971). This hypothesis states that nitrate reduction and subsequent ammonium assimilation act as a preferred photosynthate sink, thus preventing the establishment of symbiotic structures.

In order to establish whether nitrate inhibition was the result of nitrate utilization (carbohydrate deprivation and generation of combined nitrogen) or nitrate controlled physiological switches, such as nitrate reductase, Carroll and Gresshoff (1983) used a split root system of white clover on compartmentalized petri plates (Y-plates). Only one side of the plate received nitrate; both sides were inoculated with *Rhizobium*. They found that:

1. Inhibition of nodulation was localized to only that part of the root in direct contact with nitrate.
2. Nodulation continued to occur in the nitrate free portion of the root but had a lower nitrogenase activity,
3. Roots on the nitrate treated portion were generally larger than those on the nitrate free side.

This data confirms soybean results obtained by Hinson (1975). Apparently, the plant preferentially assimilated the nitrate in the medium thus preventing the establishment of "normal symbiotic structures". According to the carbohydrate deprivation hypothesis, nitrate reduction and ammonium assimilation act as the preferred photosynthate sink, accounting for the larger root system. Also, as the nitrate treated root was acting as a photosynthate sink, it is likely that there would be a systemic inhibition of nitrogenase activity on the nitrate free roots. However, Carroll and Gresshoff (1983) stated that the systemic carbohydrate deprivation hypothesis is insufficient in its explanation and suggests that some other factor(s) may be playing a major role in these phenomena, such as a post nitrate reduction compound (e.g. ammonium, glutamine), nitrate itself (analogous to and possibly simultaneous in its role of inducing nitrate reductase activity), or nitrate reductase having a regulatory role in addition to its catalytic role). Isolation of bacteroids from NO_3 inhibited soybean nodules also showed that nitrogenase activity is maintained in the isolated bacteroid but lowered in the whole nodule (Carroll *et al.* 1984). Recent work by Carroll *et al.* (1987) offers an explanation for this effect, suggesting that NO_3^- limits the oxygen availability to the bacteroids after short term NO_3^- treatments.

It is of note here that although ammonium may be implicated in the effect of nitrate inhibition of nitrogen fixation, it has not been borne out by experimental evidence as Sandemann and Gresshoff (1985) have shown that ammonium has no effect on the activity of nitrogenase which has been partially purified from siratro derived bacteroids *Bradyrhizobium* strain ANU289. This can be explained by the fact that bacteroids lack an ammonium uptake system. (Bergersen and Turner 1967; O'Hara and Daniel 1985; Howitt *et al.* 1986).

1.4 INTERNAL OR AUTOREGULATORY FACTORS AFFECTING NODULATION.

The third level of regulation is dependent on the host plant, which mediates an internal or autoregulatory response in regard to the number, pattern and size of nodules which form on its roots.

Simply stated, autoregulation is the process whereby root cells which have been infected by *Bradyrhizobium*, and thus have become committed to nodule development, send a signal to onto-genetically newer root tissues to prevent further nodule development (see Pierce and Bauer 1983; Kosslak and Bohlool 1984; Gresshoff and Delves 1986).

This feedback mechanism allows a sufficient amount of nodulation to occur to support nitrogen fixation without an excessive usage of photosynthate or other plant resources. Phyto-hormones have been implicated in maintaining the high degree of organization, gene expression, metabolism and cellular integrity needed to carry out the the initiation, development and functioning of nitrogen fixing nodules (Letham *et al.* 1978). A summary of the evidence which implicates the involvement of phyto-hormones is included in the introduction to chapter 4 (section 4.1) and therefore will not be discussed in any detail in this chapter.

This thesis focuses on the possible mechanisms of soybean nodule autoregulation. As nitrate has been shown to inhibit the symbiosis (see section 1.3), the involvement of nitrate in the nodule autoregulation process is specifically dealt with. The following section outlines the history of nodule autoregulation studies on legumes.

1.4.1 NODULE AUTOREGULATION STUDIES

The first observations which implicated a nodule autoregulation system came from Nutman in the late 1940s, who showed that ineffective nodules, that is, nodules that are not actively fixing nitrogen, are normally found in greater numbers than effective nodules (Nutman 1949) suggesting that nodule development is regulated by substances present in effective nodules.

Similarly the fact that nodules are often formed in groups, and removal of the first formed nodules stimulated further nodulation, provided evidence for the operation of a feedback mechanism of control. Several years later, Nutman (1952) showed that the removal of root tips or nodules of *Trifolium praetense* (red clover) caused a significant transient increase in the rate of subsequent nodule formation, with a slightly higher stimulation following nodule excision than root tip extension.

This data suggested that factors produced by the root meristem, or existing nodule meristems, controlled (or "autoregulated") the further development of nodules, and so displayed characteristics commonly associated with phyto-hormones (such as in the release of lateral buds from apical dominance). As a result, the level of hormones in root nodules as compared with the surrounding tissue; the level of hormone degrading enzymes in the nodules; and the effect of exogenously applied plant hormones on nodule development have been studied to varying extents. However, the conclusions which can be drawn offer no clear function for plant growth regulators in controlling any aspect of nitrogen fixation (this data is discussed in Chapter 4).

Further evidence for the operation of a feedback mechanism of control has been presented by Bhuvaneswari *et al.* (1980) who used growth pouch assays to investigate the early events in the infection of soybean (*Glycine max*) by *B. japonicum*. It was stated that the host response leading to infection and nodulation is initiated in less than two hours after inoculation. They further suggested that the diminished frequency of nodulation might be a host mediated response which could serve to prevent over-nodulation.

Experiments using double inoculation done by Pierce and Bauer (1983) showed that the number of nodules which develop on the primary root of soybean seedlings (*Glycine max*) after inoculation with *Bradyrhizobium japonicum* is substantially diminished in the region maximally susceptible to nodulation at the time of inoculation. This rapid inhibition of nodulation was investigated by inoculating soybean seedlings with rhizobia at two different times, 15 hours apart. Living strains, but not heterologous or UV killed rhizobia were capable of eliciting a rapid regulatory response. These results also showed that the diminished nodulation resulting from the delayed inoculation was an actual inhibition of nodule formation, rather than an artifact resulting from insufficient numbers of infective rhizobia in contact with the younger portion of the root.

Pierce and Bauer's results suggest that the diminished frequency of nodulation is due to a fast-acting regulatory mechanism in the host which could prevent excessive nodulation. In addition, they stated that this regulatory response may be an important factor contributing to the clustering of nodules in the crown region of the soybean root in field grown plants and for the sparse nodulation commonly observed in younger regions of the root.

Since nodulation was suppressed in portions of the root just 10 - 15 hours younger than the first inoculated portion, Pierce and Bauer (1983) argued that the inhibitory response was elicited and expressed during the 24 hour interval between inoculation and infection thread formation. However, more recent work by Calvert *et al.* (1984) in which

they performed serial sections of infections (generated by "spot" inoculations) on 70 mm root sections of soybean seedlings, demonstrates that the original hypothesis was wrong and that nodule formation appears to be suppressed at the stage of nodule emergence rather than at the stage of root hair infection. In addition, Heron and Pueppke (1987) repeated the delayed inoculation experiments of Pierce and Bauer but could not demonstrate the reported inhibition of nodulation resulting from two inoculations 15 hours apart.

The theory of a feed-back mechanism of control is also supported by the work of Kosslak and Bohlool (1984), and is consistent with the results from Calvert *et al.* (1984). This work demonstrates a nodule suppression system which requires several days, and not merely hours, to manifest itself. However, it must be noted that if these two observations are correct (i.e. 15 - 24 hour response versus a response taking several days) then they may represent two different phenomena.

Kosslak and Bohlool (1984) showed that in a split root system of soybean (*Glycine max*), inoculation of one half-side suppressed subsequent development on the opposite side. In a short day season, nodulation by the secondary inoculum was inhibited by 100 % when inoculation was delayed for 10 days. Similarly, nodulation on the second side was significantly suppressed when the secondary inoculum was delayed for only 4 days. Nodule suppression on the second side was not related to the appearance of nodules or nitrogenase activity on the side of the split roots which were inoculated at time zero. (Suppression of nodulation had already occurred in those treatments in which the introduction of the rhizobia was delayed for 4 days. This was 4 days prior to the appearance of nodules and 8 days days prior to the detection of significant nitrogenase activity which received the primary inoculum at time zero).

The most recent work supporting the notion of an autoregulatory system in soybean comes from Delves *et al.* (1986) who conducted reciprocal grafts with the wild type parent cultivar Bragg. They suggest that the loss of the autoregulation character in the super-nodulating soybean mutants (described in section 1.6 below) may be caused by the failure of the mutant to produce an inhibiting substance that is usually present in the wild type. However, from their data it is unclear whether this is the case or whether the mutant condition is due to an enhanced nodulation factor.

Delves *et al.* (1987a) also stated that all of the 9 nitrate tolerant mutants tested by means of wedge grafts, display shoot control of the super-nodulation phenotype (see section 1.5.2 and table 3.1), and further, that it is possible to differentiate between mutants in which the autoregulation response is completely suppressed, and those in which suppression is only partial.

Thus the accepted view at the initiation of this thesis was that: (1) soybean nodulation is regulated by a rapid, (within 15 hours) and possibly by a delayed (between 4 and 10 days) feedback mechanism of control; (2) a graft transmissible shoot factor is involved in nodule autoregulation; (3) phyto-hormones may be directly involved in the autoregulation response.

1.5 HOST GENETIC STUDIES

The *Rhizobium* symbiont has generally been used in studies to overcome nitrate inhibition (Gibson and Pagan 1977; Streeter 1982; McNeil 1982). Although some of these studies have been useful in highlighting the host's contribution to the symbiosis (Halverson and Stacey 1984,85), they generally demonstrated the futility in searching for *Rhizobium* strains for nitrate tolerant symbioses. The isolation of nitrate tolerant legume hosts is more promising.

One such study by Jacobsen (1984) indicated that nitrogen fixation appears to be nitrate tolerant in a nitrate reductase deficient pea mutant. Also, differential nodulation tolerance has been demonstrated between species and between cultivars within a species (Harper and Gibson 1984 a,b; Carroll *et al.* 1984; Gibson and Harper 1985).

Carroll and Gresshoff (1986) have suggested that nitrate inhibition of the early stages of nodulation appears to occur prior to the reduction of nitrate in the host tissue since the nitrate reductase deficient pea mutant E1 is nitrate tolerant for nitrogen fixation (Feenstra *et al.* 1982) but not for nodulation (Jacobsen 1984).

Carroll (1985b) also isolated 15 independent nitrate tolerant mutants of soybean (cultivar Bragg) that not only show nodulation tolerance to exogenous nitrate but also produce nodule numbers far in excess of the wild-type parent cultivar. These mutants were designated nts for nitrate tolerant symbiosis and are commonly referred to as "super-nodulating"

These nitrate tolerant mutants, particularly nts382 have been chosen for use in comparative studies with the wild-type soybean (cv. Bragg) as they not only offer the opportunity to elucidate the mechanism by which nitrate inhibits the initiation and development of nodulation, and symbiotic function, but also allow an exploration of the possible mechanism(s) of nodule autoregulation, since these mutants are defective in the control mechanism (Carroll *et al.* 1985 a,b).

1.5.1 ISOLATION OF NTS MUTANTS

Soybean seeds of *Glycine max* (L) Merr. cv. Bragg were mutagenized (see Carroll *et al.* 1985b) with ethyl methane sulfonate (EMS) (either at 0.44 % for 4 hours or 0.50 % for 6 hours). The M_2 progeny (that is the first generation after mutagenesis) were screened for increased nodulation under high nitrate conditions. Twelve seeds from each of several thousand families were planted at a 2 cm depth in pots of river sand (25 cm diam. and 25 cm height) and were inoculated with *Bradyrhizobium japonicum* strain CB1809. The plants were grown for 5 - 7 weeks in the presence of 5 mM KNO_3 in a nutrient solution as used by Herridge (1977), except that all the nutrients except $CaCl_2$ and KNO_3 were administered at quarter strength for the first two weeks and full strength thereafter. The nitrate concentration used (5 mM) was chosen on the basis of a nitrate concentration versus nodule dry weight profile obtained with the parent cultivar Bragg. The plants were grown outside during summer and inside glasshouses during winter, spring and autumn. The min. and max. summer temperatures were 12.4°C - 26.9°C and glasshouse temperatures were held between 14 - 30° C. After 5 - 7 weeks the plants were removed from the sand and screened for the extent of nodulation.

1.5.2 CHARACTERIZATION OF THE SOYBEAN MUTANT NTS382

1.5.2.1 Infection

Bauer (pers. comm.) demonstrated that both nts382 and Bragg were infected by the same mechanism, however, the number of infection events (pseudo-infections and infection thread structures) was higher in nts382, and the region of infectibility was maintained for a longer period.

1.5.2.2 Nodulation

Under all conditions tested, 4 week old nts382 plants had considerably more nodules than wild type Bragg plants grown on 5.5 mM KNO_3 or 5.5 mM urea. In the parent cultivar, all nitrogen sources tested (KNO_3, urea, NH_4Cl, NH_4NO_3) reduced nodule number. In contrast, mutant line nts382 grown on the same nitrogen sources had increased nodule numbers per plant over that of the KCl controls (Carroll *et al.* 1985a) (see fig 1.7, 1.8 and 1.9 for comparison of nodulation patterns).

1.5.2.3 Nitrogenase activity

In Bragg, supplementing the nutrient media with nitrate or urea caused a substantial reduction in nitrogenase activity of four week old plants (as measured by the acetylene reduction assay). In contrast, 2.75 mM KNO_3 significantly stimulated acetylene

Figure 1.7 **Nodulated root system of the wild-type soybean (*Glycine max* [L.] Merr) cv. Bragg**

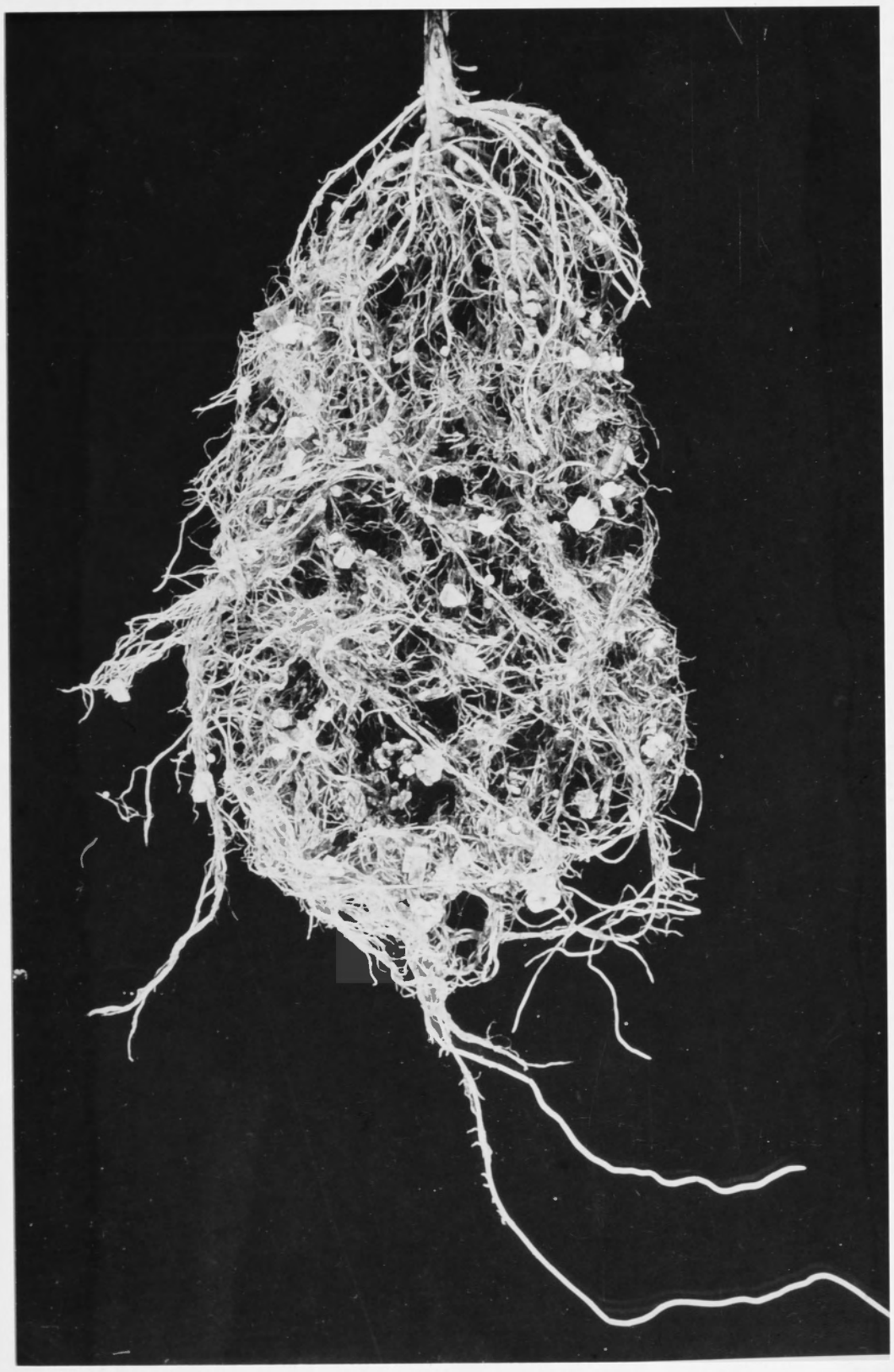


Figure 1.8 **Root system of the super-nodulation soybean mutant nts382**

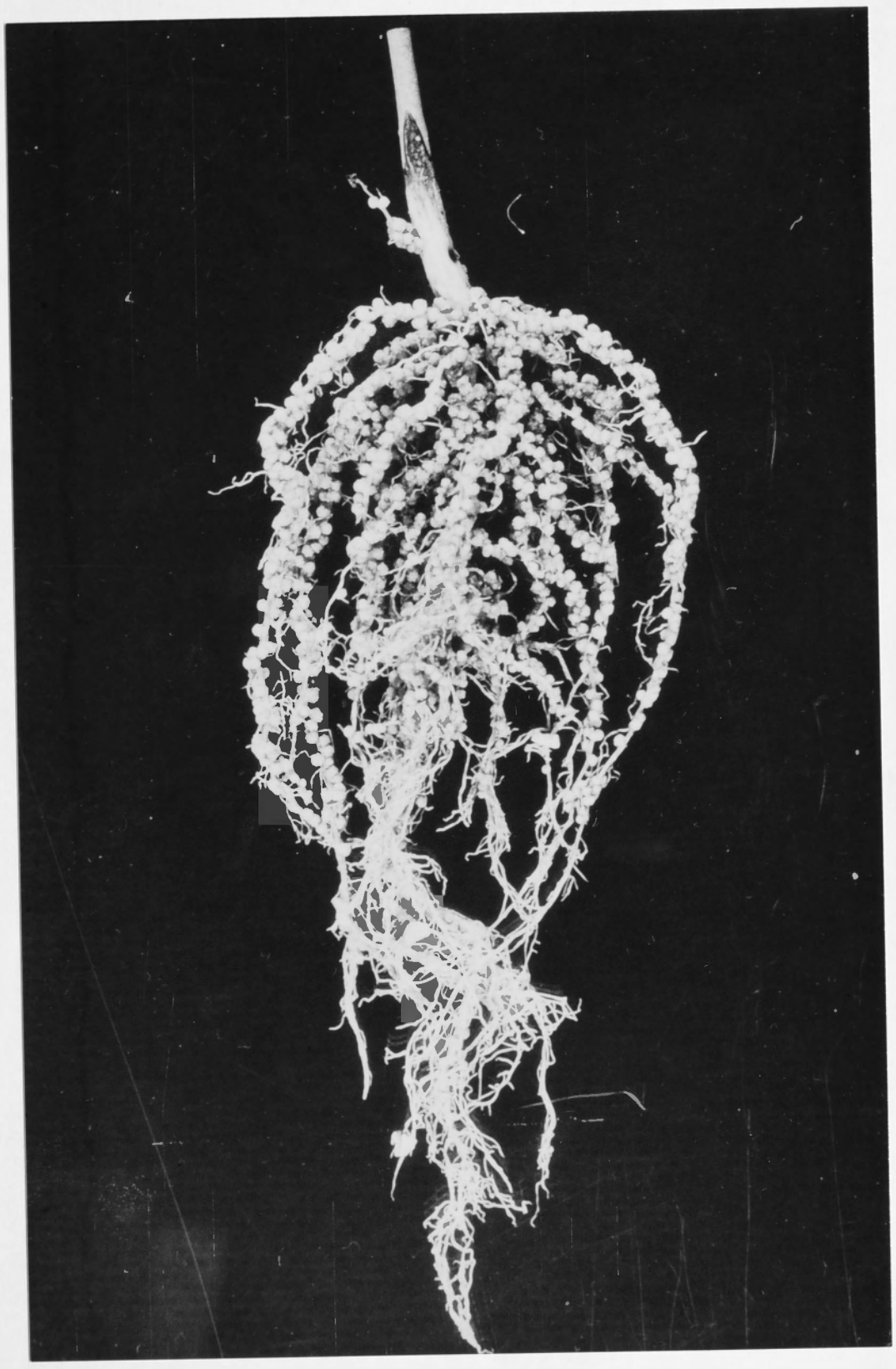


Figure 1.9 **Nodules on the nts382 mutant, showing the super-nodulation phenotype**

reduction activity per gram plant fresh weight in nts382. However, nitrogenase activity for nts382 on 5.5 mM KNO_3 was not significantly different for nitrogen free plants (Carroll *et al.* 1985a). Nitrogenase activity per plant fresh weight in four week old nts382 plants cultured on 2.75 mM and 5.5 mM KNO_3 was significantly higher than for Bragg plants cultured in the absence of nitrogen. Specific nitrogenase activity in nts382 nodules was lower than in Bragg nodules. This was explained by a lowered bacteroid concentration per nodule and in part reflects the altered nodule morphology of an nts382 nodule compared to a Bragg nodule (see also Day *et al.* 1987).

1.5.2.4 Plant growth comparisons

Studies by Day *et al.* (1986) on the comparative growth status of Bragg and nts382 plants (grown under nitrate free conditions), 13 days after sowing, (i.e. nodules were just beginning to appear), show that the shoot, root , and total plant dry weight as well as leaf area and N content of seedlings were greater in the nts382 mutant but that the shoot : root ratio in the mutant was smaller.

In particular, lateral root development in the mutant was prolific. The length of the tap roots was virtually the same as Bragg, therefore, nts382 had more lateral roots per unit length of tap root than did Bragg. (Similar results have been obtained with a super-nodulation mutant of pea as shown by Jacobsen 1984). This characteristic indicates that growth regulators may be involved in the super-nodulation phenotype (see Chapter 4 - Effect of applied growth regulators on soybean nodule and plant growth of super-nodulation and wild-type soybeans).

To test whether the slower growth of inoculated nts382 was due to its super-nodulation phenotype or to some other effect of the mutagenesis process Day *et al.* (1986) grew plants, un-inoculated in sterile vermiculite and watered with a nutrient solution containing 5 mM KNO_3. In contrast to inoculated plants, un-inoculated nts382 growth was similar to that of cv. Bragg. The shoot dry weight and leaf area as well as N content on a whole plant basis were not significantly different, but root dry weight increments however were less in nts382, resulting in a higher shoot : root ratio in nts382 in later growth stages.

Thus, the overall difference in growth of inoculated plants was attributed to super-nodulation of nts382. The similar growth of nts382 and Bragg on an external N source, together with similar plant N content confirms Carroll *et al.*'s (1985 b) previous indications that nitrate metabolism and uptake by these plants are essentially the same. A summary of studies by Day *et al.* (1986) on the physiology of nts382 in relation to cv. Bragg are shown in Table 1.1.

Day *et al.* (1986,1987) state that in general, nodules of super-nodulated nts382 plants resemble under-developed cv. Bragg nodules. When super-nodulation was avoided by using low inoculum doses nts382 nodules resembled those of cv. Bragg (Schuller *et al.* 1986; Gresshoff *et al*, 1987). Carroll *et al.* (1985b) have suggested that nts382 is a mutant in the regulation of nodule initiation and nodule development, and the autoregulation mechanism normally limiting nodulation in the wild type soybean is anomalous. In nts382 mutant plants, the super-nodulation phenotype corresponds with the diminished autoregulation response. Therefore, experiments with nts382 should help elucidate the mechanisms of nitrate inhibition and autoregulation of soybean nodulation.

1.5.2.5 Genetics of nts mutants

Genetic analysis was done on a number of the nitrate tolerant super-nodulating (nts) mutants of the wild-type soybean *Glycine max.* cv. Bragg by Delves *et al.* (1988). They demonstrated that all of the nts mutants except nts1116 were isolated as the result of a mutational event in a single locus, following chemical mutagenesis. All of the mutants except nts1116, fell into the same complementation group. The two nodulation mutants rj_1 and nod139 (tentatively designated rj_6) were able to epistatically suppress the super-nodulation phenotype.

Table 1.1 Comparison of nts382 and cv. Bragg - growth and nodule physiology (after Day *et al* 1986)

Nodule number :.....	Greater in nts382 than Bragg
Nodule mass :.....	Greater in nts382 than Bragg
Individual nodule mass :.....	Less in nts382 than Bragg
Bacteroid content :.....	Less in nts382 than Bragg
Haem content :.....	Less in nts382 than Bragg
Acetylene reduction/ mg. bacteroid protein :.....	Same in nts382 and Bragg
Ratio of infected cell to total nodule area :.....	Smaller in nts382 than Bragg
Bacteroids/ peri-bacteroid envelope :.....	Fewer in nts382 than Bragg
Nodule carbohydrate content :.....	Similar in nts382 and Bragg
Nodule and xylem sap ureides :.....	Higher in nts382 than Bragg

1.6 STATEMENT OF APPROACH

The aim of this thesis is to investigate further the control of nodulation in soybean, and in particular, the involvement of systemically translocated factors in autoregulation. The nitrate tolerant and super-nodulating mutant has been used in comparison with its wild-type parent cultivar cv. Bragg in all experiments to aid in this investigation.

Chapter 1 is designed to introduce the reader to the legume - *Rhizobium* symbiosis and in particular to the regulation of nodule development within that system. Attention is drawn to the areas of internal or autoregulation of nodulation and the regulation of the symbiosis by nitrate, and to the existance of soybean mutants that are defective in both of these control mechanisms.

Chapter 2 is a brief chapter which outlines the general materials and methods relevant to the entire thesis. Other chapters may contain specific materials and methods relevant to that particular study.

Chapter 3 is entitled "Grafting studies and evidence for the synthesis of a nodulation inhibitor in the wild-type soybean shoot" and begins with a reiteration of data from Delves *et al.* (1987 a,b) which demonstrates that the autoregulation factor is shoot controlled and systemically translocated. The grafting experiments were an attempt to discover whether autoregulation is caused by a positive signal in the Bragg plants and absence or diminution in the super-nodulation mutants, or alternatively, whether super-nodulation is caused by a nodule enhancement factor which is permanently turned on in the mutant. The "challenge" experiment continued on this theme and examined the time course of autoregulation. The reliability of transmission of compounds across the graft union was tested by injecting radio-labelled 2,4-D. Approach grafts, in which one shoot was removed either 2 days before or after inoculation were used to investigate the effect of a single shoot (wild-type or mutant) on the root systems.

Chapter 4 is entitled " effects of applied growth regulators on nodulation and plant growth of super-nodulation and wild-type soybeans" and follows the theme of nodule autoregulation and investigates the effect of exogenous application of some phyto-hormones and growth regulators on the nodulation and plant growth of the wild type soybean cv. Bragg and its super-nodulation, and nitrate tolerant mutant nts382. The interaction of nitrate and phyto-hormone action is investigated by growing plants in either the absence of nitrate or in the presence of 0.5 mM or 5.5 mM KNO_3.

Chapter 5 is entitled "host genetic control of soybean nodulation in split root systems" and returns to systemic communication experiments with the use of a split root

system of soybeans. In this chapter, experiments were done to determine the time course of autoregulation and the effect of nitrate on the interpretation of split root data. The shoots of autoregulated plants were labelled with sucrose to investigate its partitioning to the roots and to see whether nodulated (and hence fixing) roots provide a greater sink for photosynthate than non - nodulated roots.

Chapter 6 integrates the significance of the findings of this thesis with conclusions from previous investigations (as reported in the literature and by personal communications) and proposes models of soybean nodule autoregulation, and nitrate inhibition of nodulation. This conclusion chapter also outlines the scope for future experiments in this field.

CHAPTER TWO

MATERIALS AND METHODS

This chapter contains the common materials and methods used throughout the thesis. However, individual chapters contain specific techniques or procedures relevant to that chapter when required.

2.1 PLANT MATERIAL

The two lines of soybean (*Glycine max*. L. Merrill) used consistently throughout this study were the wild-type Bragg, and its nitrate tolerant "super-nodulating" mutant nts382. Bragg seed was originally supplied by Dr. David Herridge (NSW Dept. of Agriculture, Tamworth). The nitrate tolerant mutant nts382 was isolated by Carroll *et at.* (1985b) of this department.

Also, the intermediate nodulators nts1116 and nts1007, which were used in some experiments shown in chapters 3 and 5 were isolated by Carroll *et al.* (1985a,b). Three wild-type cultivars of soybean other than Bragg were used in chapter 5, namely Williams, Clarke and Lee, all of which were grown for seed in the Botany Dept. glasshouses, A.N.U.

2.2 *BRADYRHIZOBIUM JAPONICUM* STRAINS

The slow growing *Bradyrhizobium japonicum* strain USDA110 was used throughrout this study as the inoculant strain. It was originally supplied by Dr. John Streeter (Ohio State University, Wooster, USA).

2.3 PLANT CULTURE PROCEDURE

2.3.1 NON - STERILE TECHNIQUE

Seeds were planted at depth of approximately 3 cm in plastic pots (15 cm diam. x 15 cm height) containing a layer of gravel (for drainage) which was overlaid with a mixture of 3 : 1 river sand to vermiculite as a growth medium.

The nutrient solution used was based on that of Herridge (1977) and was administered at one - quarter strength (except for $CaCl_2$ which was kept at full strength) during the first two weeks of plant growth in pot experiments and at full strength thereafter. The composition of full strength Herridge stock solutions and the volumes required to prepare both one-quarter and full strenth solutions are shown in Table 2.1. Potassium nitrate (KNO_3) was added to the nutrient solution at either 0.5 mM or 5.5 mM as required.

Plants were grown in a glasshouse with average temperatures of 19° -30° C and average light intensity of 650 µE m. sec^{-1}. The latitude and longitude were 35° 17' south 149° 08' east.

2.3.2 STERILE TECHNIQUE

Seeds were often inoculated at the time of planting with *B. japonicum* cultured in Bergersen's Modified Medium (BMM, see table 2.2) or from peat cultures (see section 2.4: Preparation of *B. japonicum* strains for inoculation), however some experiments required the pots to remain *Rhizobium* free until the seedlings had emerged and the cotyledons had opened. To ensure this, seeds were surface sterilized by rinsing in 70 % ethanol for 1 minute, soaked in 3 % sodium hypochlorite for 3 minutes, and rinsed 10 times with sterile distilled water. Pots were washed in a 3% solution of sodium hypochlorite prior to pot fill and the river sand was autoclaved at 120° for 20 minutes prior to mixing with vermiculite. Pots were taped to prevent leakage of sand from the drainage holes, rather than using gravel which would otherwise have to be autoclaved.

2.4 PREPARATION OF *B.JAPONICUM* STRAINS FOR INOCULATION

2.4.1 CULTURE WITH BERGERSEN'S MODIFIED MEDIUM (BMM)

B. japonicum strains USDA 110 was cultured and stored on Bergersen's Modified Medium (BMM) plates (Bergersen 1961). Liquid cultures of *B. japonicum* were produced by taking a loopful of colonies from a BMM plate and transfering them into 100 ml of BMM culture in a Klett flask. The flask was incubated at 28° C on a rotary shaker for ± 6 days or until log phase. Approximately 10^9 cells / ml were applied to each pot as an inoculum.

The growth of liquid cultures could be continuously measured following the increase in turbidity as measured by a Klett Summerson photoelectric colourimeter using a 540 nm green filter. Units of measurement are simply referred to as "Klett number".

Alternatively *B. japonicum* strains were streaked on BMM plates which were sealed with a strip of Nescofilm (Nippon Shoji Kaishi, Japan) and incubated inverted in the 28°C constant temperature room. The composition of BMM is listed in table 2.2 and trace elements, which are a component of BMM are listed in Table 2.3.

Contamination of cultures was checked by streaking on LBG plates (see Table 2.3)

2.4.2 PREPARATION OF PEAT CULTURES OF *B. JAPONICUM*

Sterilized bags of dry peat (40 g bags) were obtained from Agricultural Laboratories Pty. Ltd., Regents Park NSW.

45 ml of *B. japonicum* BMM liquid culture (see 2.4.1) was injected into the peat bags which were subsequently incubated at 28° C for 10 to 14 days. After incubation the bags contained 10^9 *B. japonicum* cells / g. (as confirmed by colony counts from serial dilutions plated onto BMM plates).

Mature peat cultures were stored at 4° C until use. The inoculant was supplied to pots as a slurry (peat culture mixed with nutrient solution). Each pot recieved approximately 10^8 - 10^9 cells at planting or after cotyledon emergence (depending on experimental design).

2.5 SCINTILLATION MIX FOR LIQUID SCINTILLATION COUNTING.

Material was prepared as described in the experimental outline of individual chapters, and then samples were left overnight in 10 ml of scintillation mixture:

1.5 l Toluene,
1 l Triton X,
40 ml Permafluor,
25 ml NCS tissue solubilizer pH 7.0

Radioactivity was measured in a Beckman LS 7000 scintillation counter and expressed in counts per minute (cpm).

2.6 Chemicals

Chemicals used throughout this study were obtained from either Sigma Chemical company (Missouri USA); Ajax (Australia); or BDH (Australia).

Table 2.1 HERRIDGE PLANT NUTRIENT SOLUTION (Herridge 1977)

STOCK SOLUTIONS		1/4 Strength (mg/l)	Full Strength (mg/l)
1. **Salt stocks**[1]	(1 M)		
$MgSO_4.7H_20$	(138 g/l)	0.12	0.50
$CaCl_2$	(147 g/l)	0.25	0.25
K_2HPO_4	(174 g/l)	0.015	0.06
KH_2PO_4	(136 g/l)	0.015	0.06
KCl	(75 g/l)	0.06	0.25
2. **Ferric monosodium salt of EDTA**	(34.5 g/l)	0.06	0.25
OR $FeSO_4.7H2O$ [2]	(2.78 g)		
Na2.EDTA	(3.72 g)		
d.H20	500 ml		
3. **Trace Elements** [3]		0.06	0.25
$MnSO_4.H_2O$	100 mg		
H_3BO_3	30 mg		
$ZnSO_4.7H_2O$	30 mg		
$Na_2MoO_4.2H_2O$	2.5 mg		
$CuSO_4.6H_2O$	2.5 mg		
$Co.Cl_2.6H_2O$	2.5 mg		
d. H_2O	100 ml		
0.5 mM KNO_3		0.09 g	0.09 g
5.5 mM KNO_3		0.50 g	0.50 g

1 Each salt solution was prepared and stored separately at 4°C

2 Heat to dissolve

3 Trace elements were collectively prepared, filter sterilized and stored at 4°C

Table 2.2 BERGERSEN'S MODIFIED MEDIUM (Bergersen 1969)

	STOCK SOLUTIONS	Stock	For 1 l. solution	Final concentration (mg / l)
1.	**Na_2HPO_4. $12H_2O$**	45 g /1	8 ml	360
2.	**$MgSO_4$. $7H_2O$**	10 g / 1	8 ml	80
3.	**$FeCl_3$. $6H_2O$**	20 g / 1	0.15 ml	3
4.	**$CaCl_2$. $2H_2O$**	40 g / 1	1 ml	40
5.	**Biotin**	100 mg / 100 ml	1 ml	1
6.	**Thiamine**	10 mg / 100 ml	1 ml	0.1
7.	**Trace Elements**		1 ml	
	$MnSO_4.H_2O$	100 mg		1
	H_3BO_3	30 mg		0.3
	$ZnSO_4$. $7H_2O$	30 mg		0.3
	$Na_2MoO_4.2H_2O$	2.5 mg		0.025
	$CuSO_4.6H_2O$	2.5 mg		0.025
	$Co.Cl_2.6H_2O$	2.5 mg		0.025
8.	**Sodium glutamate**	0.5 g		500
9.	**Mannitol**	10 g		10,000
10.	**Yeast Extract**	0.5 g		500
11.	**d. H_2O**	980 ml		

BMM AGAR PLATES

The composition of BMM agar plates was the same as the liquid except that the concentration of mannitol was 3 g / l (instead of 10 g / l.). The agar concentration was 15 g / l.

Table 2.3 LURIA BROTH + GLUCOSE

Peptone	10 g	
NaCl	5 g	
Yeast extract	5 g	
Glucose	5 g	
Agar	10 g	
$d.H_2$	1000 ml	pH 7.0

Autoclave at 20 p.s.i. for 20 minutes.

CHAPTER THREE

GRAFTING STUDIES AND EVIDENCE FOR THE SYNTHESIS OF A NODULATION INHIBITOR IN THE WILD-TYPE SOYBEAN SHOOT

3.1 INTRODUCTION

It is well known that in grafts of higher plants, an exchange of various substances takes place between the scion and stock. The mechanism of translocation between the cells of both partners - symplastic or apoplastic, is however an unresolved question (Kollmann and Glockmann 1985).

Grafting studies have been previously made to investigate the legume *-(Brady)-rhizobium* symbiosis, into such areas as the non-nodulating character in soybeans (Tanner and Anderson 1963b); the specificity of combinations of genotype and *Rhizobium* strain (Caldwell *et al.* 1966); and the relative importance of root and shoot factors on the soybean symbiosis (Lawn and Brun 1974a,b; Lawn *et al.* 1974; Lawn and Bushby 1982).

Work done in the Botany department (ANU) has used nitrate tolerant mutants and wild-type soybeans to demonstrate that super-nodulation and hyper-nodulation is shoot controlled in the mutants nts382 and nts1116 (Gresshoff and Delves 1986, see table 3.1). Further they showed that in reciprocal root : shoot grafts that shoot control of super-nodulation can be induced on another legume species (*G. soya*) by a mutant of *G. max* (nts382). They concluded that a similar method of chemical signalling may be used to induce nodulation control amongst legumes (Delves *et al.* 1987 a,b)

Previous experiments also done by this group have shown that grafting above or below the cotyledon had no effect on the nodulation or plant growth response. These results show that the cotyledons are not involved in the above phenomenon.

Streeter (1974) reported a method of approach grafting as a method for testing the nitrogen supplying capacity of nodulated soybean roots, in which one root system was cut from the grafted plants, thus doubling the shoot : root ratio. In the period 2 - 21 days after doubling the shoot : root ratio, both root and nodule dry weight and acetylene reduction increased relative to grafted plants with only one shoot and root. Their results indicated that nodulated *G. max* cv. Harosoy 63 plants are capable of fixing nitrogen at rates greater than those which normally prevail.

Two types of grafting have been used in this study, namely the approach and wedge techniques. The distinguishing feature of approach grafts is that two independent, self sustaining plants are grafted together, whereas for wedge grafts scions and stocks from different plants are used.

Table 3.1 **Summary of grafting data with super-nodulation soybean mutants** (from Gresshoff and Delves 1986)

Graft	Nodule number	Nodule mass (mg)	Tap root length (cm)	% root nodulated	Nitrogenase activity
Bragg control	31 (11)	43 (10)	29 (5)	upper 33	1860 (360)
Bragg shoot/ nts382 root	76 (26)	45 (21)	29 (5)	upper 33	1320 (780)
nts382 control	278 (144)	151 (43)	18 (5)	95	480 (60)
nts382 shoot/ Bragg root	213 (175)	109 (41)	23 (3)	95	1080 (300)

Data in this table are means of 10 plants with the standard deviation in brackets. Nodule mass is dry weight and nitrogenase activity is expressed as nmol ethylene produced per hour per gram nodule dry weight. Nodule number and nodule mass values are expressed per gram dry weight of plant.

3.2 MATERIALS AND METHODS

3.2.1 APPROACH GRAFTING TECHNIQUE

The approach graft technique was used to investigate the effect of connecting the vascular systems of the wild-type soybean and its super-nodulating mutants on the subsequent nodulation patterns.

The two seedlings grown in each pot were grafted 10 days after planting (DAP). At the point where the union was to occur, a slice of stem, approximately 2 cm long, and extending into the vascular cylinder was cut from both stems. The cuts were made in the region 1 - 3 cm below the cotyledons and were made as smooth and flat as possible so that when pressed together there was close contact between the vascular tissues. The graft region was bound tightly with teflon tape.

Grafts were placed immediately under an intermittent automatic misting system for 5 days after which they were transfered to a glasshouse and inoculated with *Bradyrhizobium*. (see figure 3.1)

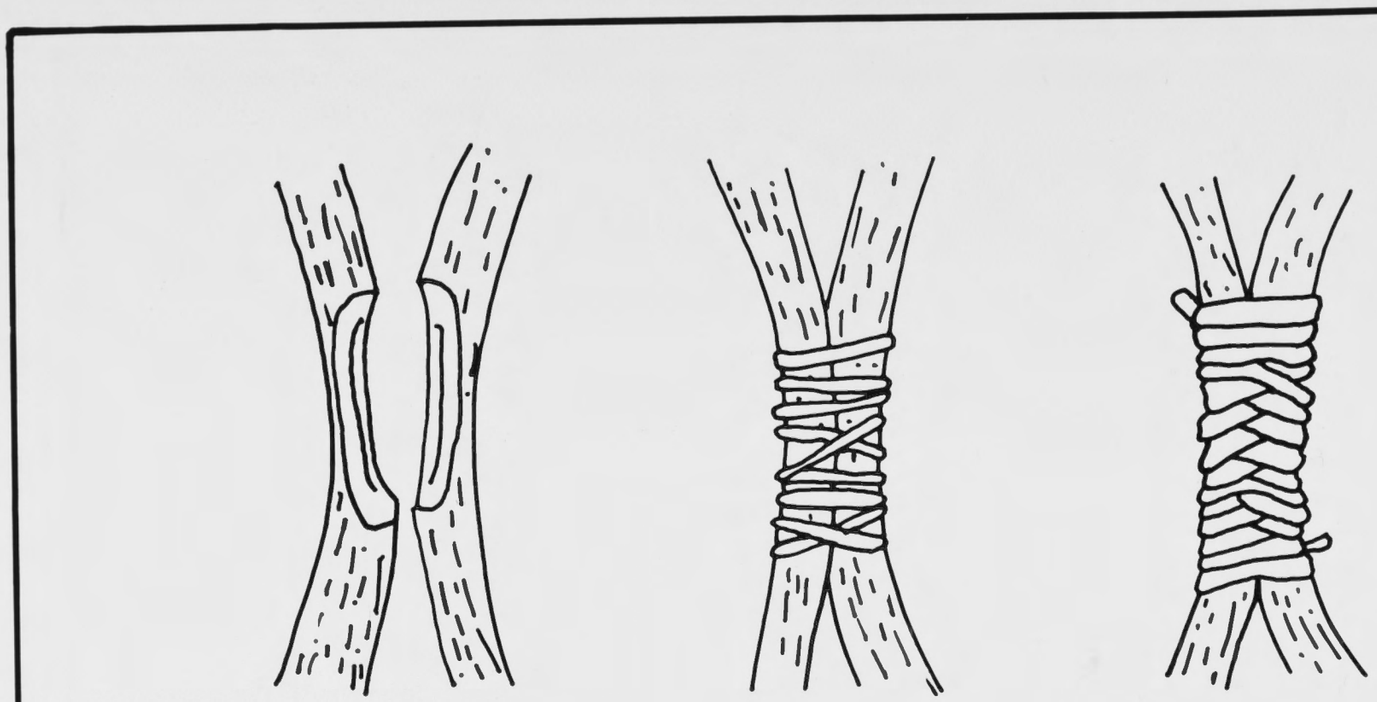
3.2.2 WEDGE GRAFTING TECHNIQUE

Grafting was conducted 10 days after planting, using a wedge shaped graft with the cotyledons left on the scion. Grafts were held in place by a polythene sleeve covering the whole graft. Grafted plants were then placed under an intermittent, automatic misting system for 5 days to prevent desiccation before the grafts had functionally joined. After grafting, primary leaves were trimmed to prevent excessive transpiration. Plants were then transfered to a glasshouse, inoculated with *B. japonicum* USDA110 as a slurry of bacteria, peat and water (approximately 10^7 to 10^8 bacteria per plant) (see figure 3.2).

Figure 3.1 **The Approach - graft technique**

Step 1: 2 cm long cuts (extending into the vascular tissue) were made on the stems of similar aged plants at the point 1-3 cm below the cotyledons.

Step 2: The stems were bound together with the wounded surfaces facing each-other.

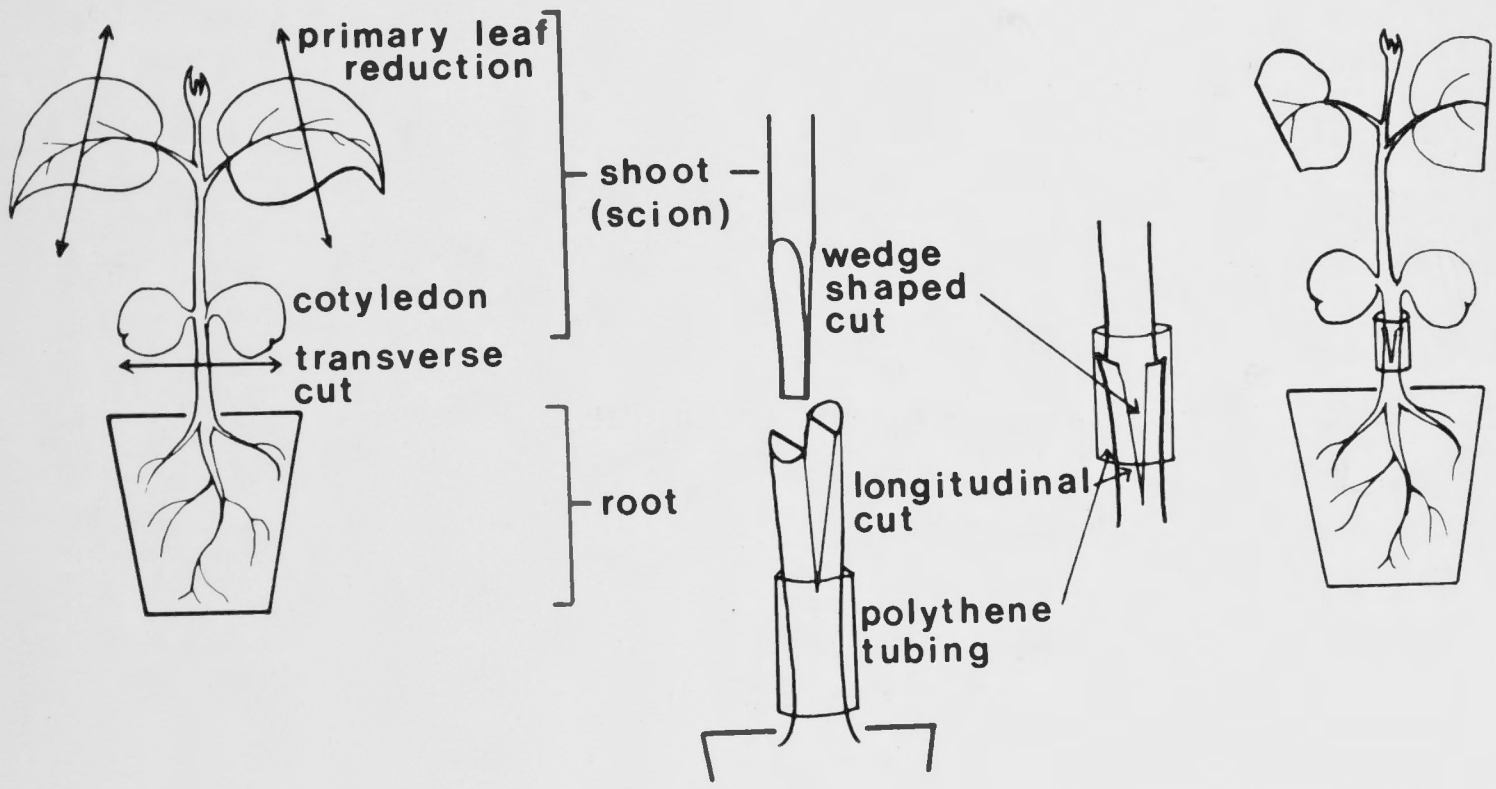


STEP ONE

STEP TWO

Figure 3.2 The Wedge - graft technique

Wedge grafting technique



DETAILED EXPERIMENTAL DESIGN and RESULTS

Experiments are numbered for ease of reference, but this bears no relation to the order of experiments performed during the thesis, nor is it meant to infer that experiments were not repeated. The data shown in experiments 1 to 15 are from typical, repeatable experiments.

3.3 EXPERIMENT 1: EFFECT ON NODULATION OF APPROACH GRAFTING NTS382 AND BRAGG SOYBEANS GROWN IN THE ABSENCE AND PRESENCE (5.5MM KNO_3) OF NITRATE.

3.3.1 INTRODUCTION

The following experiment was set up to investigate the effect on nodulation of approach grafting the nts382 mutant to its wild-type parent cultivar in the presence and absence of nitrate. In these approach grafts, the vascular systems of two plants were connected to see if the signals or substances involved in wild-type nodule regulation could be translocated to the mutant to suppress the super-nodulation phenotype. The suppression of nts382 nodulation would therefore indicate that a systemically translocatable factor was involved. Conversely, if no suppression occured it would indicate that either (a) the autoregulation signal autoregulation is not caused by an inhibitor in Bragg shoots, (b) autoregulation in Bragg is caused by a diminished ability of the wild-type to produce a nodule enhancing signal, or (c) the signal involved in autoregulation is not graft transmissable.

3.3.2 DESIGN

Seeds of *Glycine max* wild-type cultivar Bragg and its super-nodulation mutant nts382 were planted in 15 cm pots of sterile sand : vermiculite (3:1 ratio) in the following arrangements:

1 nts382 planted 2 cm away from 1 Bragg
2 Bragg seeds planted 2 cm away from each other
2 nts382 seeds planted 2 cm away from each other

Ten pots of each treatment were watered with quarter strength quarter strength Herridge nutrient solution supplemented with 5.5 mM KNO_3 and ten pots of each were watered with quarter strength nutrient solution alone. After 2 weeks, full strength nutrient solution was used.

The two plants in each pot were approach grafted ten days after planting and placed under a mist spray for 5 days to encourage the graft union. After this the pots were transfered to a glasshouse and the grafted plants were inoculated with *Bradyrhizobium* strain USDA110 (10^9 viable cells per pot). Plants were harvested 28 days after inoculation (i.e. day 43 after planting) at which time nodules were detached and counted, then dried to constant weight, along with shoots and roots at 65° C for 48 hours prior to weighing.

3.3.3 RESULTS: APPROACH GRAFTS BETWEEN NTS382 AND BRAGG

Table 3.2 shows that nts382 plants grafted to Bragg plants in the absence of nitrate had no significant difference in the number of nodules which formed on either root compared to the number which developed on the respective homo-graft. The nodule counts on the Bragg homo-graft were 130 ± 16 and 123 ± 22, while the nodule count on the Bragg side of the Bragg : nts382 graft was 161 ± 45. For the nts382 homo-grafts, the nodule counts were 548 ± 29 and 589 ± 18, while the number of nodules on the nts382 side of the Bragg: nts382 graft was 575 ± 10. These results indicate that in the absence of nitrate, the Bragg autoregulation response is not elicited.

In contrast to this are the results from nitrate treated plants. There was a significant increase in the number of nodules on the Bragg side of the Bragg: nts382 + N graft (50 ± 13) compared to either side of a Bragg: Bragg + N homo-graft (6 ± 6 and 5 ± 3). This represents an 8 fold increase in nodule number. Conversely, the nts382 nodule count on the nts382 : Bragg + N graft was significantly reduced (to 175 ± 43). This is a 3 fold decrease in nodule number compared to the number on nts382 : nts382 + N grafts (491 ± 2 and 550 ± 65). These trends are also demonstrated when nodule number is expressed per gram or root or total plant dry weight (see table 3.3).

In the absence of nitrate, plant growth rate was slower and so the graft unions may not have been completed, thus limiting the translocation of autoregulatory factors. As such both nts382 and Bragg acted as if they were un-grafted. Alternatively, in the absence of nitrate, the Bragg autoregulatory response was not elicited.

In the presence of nitrate, an intermediate result is evident. Bragg side nodulation was enhanced, while nts382 side nodulation was depleted. This may be explained by the nitrate stimulating the Bragg plant to elicit the autoregulatory signal which is then translocated across the graft union and diluted between the two plants, resulting in lowered nts382 side nodulation, but an increase in in Bragg side nodulation. Alternatively a stimulatory signal in nts382 that is absent in Bragg, is elicited in the presence of nitrate and diluted to yield the same result. It has been previously demonstrated that nitrate acts

Table 3.2 : Effect on plant growth and nodulation of approach grafts between Bragg and nts382 plants (Experiment 1)

Graft plant $^{-1}$	Nodule No. dry wt. g.	Nodule dry dry wt. g. plant^{-1}	Root dry dry wt. g. plant^{-1}	Shoot dry dry wt. g plant^{-1}
Bragg : Bragg - N				
i (Side 1) Bragg	130 ± 16	0.10 ± 0.02	0.38 ± 0.04	1.76 ± 0.04
ii (Side 2) Bragg	123 ± 22	0.10 ± 0.01	0.37 ± 0.01	1.60 ± 0.05
LSD 0.05	32			
nts382 : Bragg - N				
iii (Side 1) nts382	575 ± 100	0.17 ± 0.03	0.15 ± 0.04	1.16 ± 0.15
iv (Side 2) Bragg	161 ± 45	0.11 ± 0.01	0.42 ± 0.25	1.81 ± 0.08
LSD 0.05 between iii and v :	178			
LSD 0.05 between ii and iv :	82			
nts382 : nts382 - N				
v (Side 1) nts382	548 ± 29	0.25 ± 0.01	0.17 ± 0.01	1.60 ± 0.26
vi (Side 2) nts382	589 ± 18	0.25 ± 0.01	0.18 ± 0.01	1.50 ± 0.17
LSD 0.05	53			
Bragg : Bragg + N				
vii (Side 1) Bragg	6 ± 6	0.10 ± 0.02	0.93 ± 0.06	4.90 ± 0.38
viii (Side 2) Bragg	5 ± 3	0.10 ± 0.02	0.73 ± 0.12	4.61 ± 0.69
LSD 0.05	7			
nts382 : Bragg + N				
ix (Side 1) nts382	175 ± 43	0.04 ± 0.01	0.26 ± 0.07	1.25 ± 0.18
x (Side 2) Bragg	50 ± 13	0.03 ± 0.02	1.34 ± 0.35	3.05 ± 0.11
LSD 0.05 between ix and xi :	67			
LSD 0.05 between x and viii :	15			
nts382 : nts382 + N				
xi (Side 1) nts382	491 ± 2	0.16 ± 0.04	0.31 ± 0.01	2.75 ± 0.09
xii (Side 2) nts382	550 ± 65	0.19 ± 0.04	0.31 ± 0.01	2.79 ± 0.31
LSD 0.05	107			

Data expressed as $\bar{x}$ ± s.d; N = plus 5.5 mM KNO_3; -N = minus nitrate. Number of replicates vary between 6 and 8.

Table 3.3 : Plant growth and nodulation of approach grafts of Bragg and nts382 plants (Experiment 1)

Graft	Nodule Number / g root dry wt.	Nodule number / g total plant dry wt.
Bragg : Bragg - N		
i (Side 1) Bragg	342 ± 41	57 ± 6
ii (Side 2) Bragg	333 ± 70	59 ± 10
Nts382 : Bragg - N		
iii (Side 1) nts382	3481 ± 491	398 ± 82
iv (Side 2) Bragg	383 ± 144	68 ± 17
Nts382 : Nts382 -N		
v (Side 1) nts382	3232 ± 202	278 ± 41
vi (Side 2) nts382	3278 ± 155	303 ± 18
Bragg : Bragg + N		
vii (Side 1) Bragg	10 ± 7	1.12 ± 1.06
viii (Side 2) Bragg	8 ± 3	0.97 ± 0.39
Nts382 : Bragg + N		
ix (Side 1) nts382	637 ± 137	111 ± 17
x (Side 2) Bragg	37 ± 5	11 ± 2
Nts382 : Nts382 + N		
xi (Side 1) nts382	1613 ± 27	154 ± 6
xii (Side 2) nts382	1656 ± 188	167 ± 33

All data expressed as $\bar{x}$ ±s.d.; - N = minus nitrate; +N = plus 5.5 mM KNO_3.

via the root (Hinson 1975; Carroll and Gresshoff 1983) and so may increase the sensitivity of the root to an inhibitor or stimulator.

In the presence of nitrate it is possible that the graft anneals earlier than in its absence and, as such, Bragg autoregulatory signals would be able to pass across the graft union resulting in the observed changes in nodule number. Thus, it was considered of interest to examine the translocation of ^{14}C - 2,4-D across the graft union in the presence and absence of nitrate. The results of this experiment are shown in the next section (section 3.4.3).

3.4 EXPERIMENT 2: TRANSLOCATION OF ^{14}C 2,4-D ACROSS THE BRAGG : NTS382 GRAFT UNION

3.4.1 INTRODUCTION

Following experiment 1, it was considered necessary to investigate the reliability of the approach graft technique for translocation studies, and further, to see how a substance applied to one shoot was distributed between the two root systems.

^{14}C - 2,4-Di-chloro-phenoxy-acetic acid, a synthetic auxin (and therefore more stable than IAA) was used as the translocation indicator as it has been shown to travel in the phloem (Crafts 1956) and as such can be assayed in the roots using the liquid scintillation counting method (Yamaguchi and Crafts 1958). In a similar system, Tsurami and Wada (1980) applied ^{14}C-IAA to *Vicia faba* cotyledons and recovered 97%-98% of the total radioactivity from the ethanol fraction of the roots 8 hours later. This indicates that auxin is rapidly translocated from the cotyledon to the roots.

3.4.2 DESIGN

Approach grafts of Bragg and nts382 were set up and grown in the presence (5.5 mM KNO_3) and absence of nitrate. When the plants were 24 days old, (i.e. 14 days after grafting), 10 µl ^{14}C - 2,4-D (specific activity: 55 mCi/ mM or 2.04 GBq/mM; Total radioactivity injected: 100 nCi / plant) was injected into the Bragg stem immediately below the first trifoliolate. This procedure was done at first light, and eight hours later the roots were detached at the top of the graft junction and cut into 3cm segments. Each segment was placed in a separate PVC vial containing 10 ml of scintillation mixture and left overnight at room temperature. Radioactivity was measured in a Beckman scintillation counter and expressed as cpm per root.

3.4.3 RESULTS: ^{14}C-2,4-D TRANSLOCATION ACROSS GRAFT JUNCTIONS

Table 3.4 shows the results of translocation of ^{14}C - 2,4-D from shoots to roots in the presence and absence of nitrate in three systems: un-grafted plants, wedge grafts and approach grafts.

When labelled 2,4-D was injected into the shoots of **un-grafted** Bragg and nts382 in the presence or absence of nitrate and measured in the roots 8 hours later, there was no significant difference between the cultivars. The recovered radioactivity was measured in cpm per genotype specific side. Thus, despite the nodulation difference, there was no effect on the translocation of 2,4-D. Both nts382 and Bragg plants grown in the presence

Table 3.4: Comparison of the translocation of ^{14}C - 2,4-D in Bragg and nts382 soybeans from shoots to roots (Experiment 2)

System	Nitrate level (mM)	Recovered radioactivity (cpm / genotype specific root) $\bar{x} \pm$ s.d.
Ungrafted controls		
Bragg	0	2706 ± 1400
nts382	0	2675 ± 509
Bragg	5.5	1451 ± 154
nts382	5.5	1400 ± 421
Wedge grafts		
Bragg shoot / nts382 root	0	3439 ± 1208
nts382 shoot / Bragg root	0	3464 ± 1993
Bragg shoot / nts382 root	5.5	2731 ± 433
nts382 shoot / Bragg root	5.5	2518 ± 53
Approach grafts *		
(1) nts382 to Bragg	0	3054 (nts382) : 3843 (Bragg)
nts382 to Bragg	5.5	2218 (nts382) : 1868 (Bragg)
(2) nts382 to Bragg	0	851 (nts382) : 869 (Bragg)
nts382 to Bragg	5.5	625 (nts382) : 855 (Bragg)

All plants were inoculated and well nodulated ; Data is expressed as $\bar{x} \pm$ s.d. of 4 replicates, except for approach grafts which show two separate experiments. For approach grafts, ^{14}C - 2,4-D was injected into the Bragg shoot.

of nitrate, had a slightly lower cpm count, but due to the large standard deviation, the difference is not significant.

When Bragg and nts382 were reciprocally **wedge grafted**, and grown in the absence of nitrate there was no significant difference in the amount of label translocated from the shoots to the roots, i.e.when Bragg shoots were grafted onto nts382 shoots, 3439 ± 1208 cpm was recovered from the nts382 root, and when nts382 shoots were grafted onto Bragg shoots 3464 ± 1993 cpm was recovered from the Bragg root. The same was observed in the presence of nitrate when Bragg shoots were grafted onto nts382 roots (2731 ± 433 cpm) and nts382 shoots were grafted onto Bragg roots (2518 ± 53 cpm). These results suggest that there is no difference in the ability of Bragg and nts382 to translocate the indicator substance.

When nts382 was **approach grafted** to Bragg and ^{14}C - 2,4-D was injected into the Bragg shoot in the presence or absence of nitrate, there was no significant difference in the amount of label recovered from either the Bragg root (3843 cpm) or the nts382 root (3054 cpm), 8 hours later. In the presence of nitrate, the Bragg root accumulated 1868 cpm and the nts382 root accumulated 2218 cpm. Again there was a trend for nitrate treated plants to accumulate less sucrose in roots than those grafts grown in the absence of nitrate. These results however need to be verified with further experimentation. The repeat experiment showed a similar trend. These results demonstrated that the translocation indicator ^{14}C - 2,4-D can cross approach - graft junctions equally well in the presence and absence of nitrate. This suggests that graft junctions heal equally well in the presence and absence of nitrate. Thus it is proposed that the absence of change of nodulation on the roots of Bragg and nts382 hetero-grafts, grown in the absence of nitrate is not caused by the inability of the autoregulation signal to cross an un-healed graft junction. This suggests that the presence of nitrate in the nutrient solution has an involvement in eliciting or enhancing a basal level of autoregulation signal.

3.5 EXPERIMENT 3 : EFFECT ON NODULATION OF APPROACH GRAFTING NTS382, NTS1116 AND BRAGG SOYBEANS GROWN IN THE ABSENCE AND PRESENCE (5.5 mM KNO_3) OF NITRATE.

3.5.1 DESIGN

Wild-type soybean Bragg, and mutants nts382 and nts1116 were approach grafted as either homo-grafts, or hetero-grafts to see if the intermediate nodulator, nts1116 behaved more like the wild-type soybean or its super-nodulation mutant.

Seeds of cultivar Bragg and its super-nodulating mutants nts382 and nts1116 were planted in 15 cm pots of sterile sand:vermiculite (3 : 1 ratio) in the following arrangements:

1 nts1116 planted 2 cm away from 1 Bragg
1 nts1116 planted 2 cm away from 1 nts382
2 Bragg seeds planted 2 cm away from each other
2 nts382 seeds planted 2 cm away from each other
2 nts1116 seeds planted 2 cm away from each other

Five pots of each system were watered with quarter strength Herridge nutrient solution supplemented with 5.5 mM KNO_3 and five pots of each were watered with nutrient solution alone. After 2 weeks, full strength nutrient solution was used. The plants were grafted 10 days after planting, inoculated with USDA110 on day 15 and harvested on day 43 (4 weeks after inoculation).

3.5.2 RESULTS: APPROACH GRAFTING NTS382, NTS1116 OR BRAGG

When Bragg and nts1116 plants were approach grafted in the presence of 5.5 mM KNO_3 (see table 3.5) it resulted in the doubling of the nodule number on the Bragg root as compared to that on a Bragg homo-graft (114 ± 34 compared to 49 ± 17 respectively). No significant difference was observed on nts1116. The increased Bragg number may be due to a dilution of the autoregulation factor between the two plants, although this does not explain why nts1116 nodule number remains constant. In addition it must be considered that nts1116 may not be mutated in the same gene as nts382. These data are only included for future integration.

The opposite situation occured in the absence of nitrate (see table 3.6). When nts1116 was grafted to Bragg, there was a significant increase in the nts1116 nodule number (279

Table 3.5: Effect on nodulation of grafting the intermediate nodulator nts1116 to either Bragg or nts382 in the presence of nitrate (5.5 mM KNO_3) - Experiment 3

	GRAFT	NODULE NUMBER $\bar{x} \pm$ s.d.
	nts1116 : nts1116 + N	
i	SIDE 1: nts1116	320 ± 84
ii	SIDE 2 : nts1116	334 ± 39
	nts382 : nts382 + N	
iii	SIDE 1 : nts382	1124 ± 363
iv	SIDE 2 : nts382	1153 ± 183
	BRAGG : BRAGG + N	
v	SIDE 1 : BRAGG	51 ± 31
vi	SIDE 2 : BRAGG	49 ± 17
	nts1116 : nts382 + N	
vii	SIDE 1 : nts1116	424 ± 176
viii	SIDE 2 : nts382	1122 ± 270
	nts1116 : BRAGG + N	
ix	SIDE 1 : nts1116	490 ± 182
x	SIDE 2 : BRAGG	114 ± 34

+ N = plus 5.5 mM KNO_3
LSD 0.05 between (i) and (vii) = 179 - Not significant
between (i) and (ix) = 205 - Not significant
between (iv) and (viii) = 399 - Not significant
between (vi) and (x) = 37 - Significant difference

Table 3.6: Effect on nodulation of grafting the intermediate nodulator nts1116 to either Bragg or nts382 in the absence of nitrate (Experiment 3)

	GRAFT	NODULE NUMBER $\bar{x} \pm$ s.d.
	nts1116 : nts1116 - N	
xi	SIDE 1: nts1116	178 ± 54
xii	SIDE 2 : nts1116	174 ± 39
	nts382 : nts382 - N	
xiii	SIDE 1 : nts382	271 ± 66
xiv	SIDE 2 : nts382	252 ± 35
	BRAGG : BRAGG - N	
xv	SIDE 1 : BRAGG	74 ± 19
xvi	SIDE 2 : BRAGG	72 ± 14
	nts1116 : nts382 - N	
xvii	SIDE 1 : nts1116	207 ± 44
xviii	SIDE 2 : nts382	390 ± 82
	nts1116 : BRAGG - N	
xix	SIDE 1 : nts1116	279 ± 32
xx	SIDE 2 : BRAGG	60 ± 16

- N = absence of nitrate in nutrient solution
LSD 0.05 between (xi) and (xvii) = 77 (Not significant)
between (xi) and (xix) = 64 (Significant increase)
between (xiv) and (xviii) = 115 (Signifcant increase)
between (xvi) and (xx) = 26 (Not significant)

± 32) compared to the nts1116 homo-graft (178 ± 54 nodules). There was no significant difference in the Bragg side nodule number. As yet the increase in nts1116 nodule number cannot be explained and suggests that the molecular mechanism in nts1116 may be different from that in nts382.

When nts1116 and nts382 were grafted in the presence of nitrate, there was a slight but not significant difference in nts1116 nodulation (424 ± 176) compared to the nts1116 homo-graft (320 ± 84), and no significant difference in the nts382 nodule number. Again the opposite situation occurred in the absence of nitrate. When nts1116 was grafted to nts382 there was a significant increase in nts382 nodule number (390 ± 82) compared to that on the nts382 homo-graft (252 ± 35 nodules). No significant difference in nodulation was noted on the nts1116 root (207 ± 44) compared to that on the nts1116 homo-graft (178 ± 54). These results indicate that neither nts382 nor nts1116 possess a significant autoregulatory capacity.

3.6 EXPERIMENT 4 : EFFECT OF APPROACH GRAFTING AND THE SUBSEQUENT REMOVAL OF ONE OF THE SHOOTS ON NODULATION.

3.6.1 INTRODUCTION

Wild-type soybean (cv.Bragg) and its nitrate tolerant mutant nts382 were either homo-grafted or hetero-grafted using the approach graft technique. One of the shoots from each graft was removed either two days prior, or two days after inoculation. This was done to examine the effect of the presence of either an nts382 or Bragg shoot on nodulation of the two intact roots and the shoot growth of its graft partner. In addition another set of plants were grafted with no subsequent shoot removal. Plants were grown in the presence (0.5 mM KNO_3) and absence of nitrate to investigate its effect on nodulation patterns.

3.6.2 DESIGN

Day 0 Seeds were planted in 15 cm pots of sand : vermiculite (3:1 ratio) as follows:

1 nts382 planted 2 cm away from 1 Bragg

2 Bragg seeds planted 2 cm away from each other

2 nts382 seeds planted 2 cm away from each other

Ten pots of each arrangement were watered daily with a quarter strength Herridge solution containing 5.5 mM KNO_3 (+N) and ten pots were watered with nitrate free (-N) nutrient solution.

Day 13 Seedlings were approach grafted

Day 17 Two days prior to inoculation remove one of the shoots was removed as follows:

(i)	B: B - N	One of the Bragg shoots
(ii)	B: B + N	One of the Bragg shoots
(iii)	B : 382 - N	The Bragg shoots
(iv)	B : 382 - N	The nts382 shoot
(v)	B : 382 + N	The Bragg shoot
(vi)	B : 382 + N	The nts382 shoot
(vii)	382 : 382 - N	One of the 382 shoots
(viii)	382 : 382 + N	One of the 382 shoots

Day 19 : All grafts were inoculated with *Bradyrhizobium japonicum strain* USDA 110

Day 21 : Two days after inoculation remove one of the shoots was removed as shown in (i) to (viii) above.

Day 55 : Nodules were detached and counted. Roots, shoots and nodules were dried at 65°C for 48 hours prior to weighing.

3.6.3 RESULTS: APPROACH GRAFTING AND THE SUBSEQUENT REMOVAL OF ONE OF THE SHOOTS ON NODULATION

In the discussion of these results, chemical nomenclature involving "*cis*" and "*trans*" is used to specify shoot versus root configurations. The *cis* root refers to the one who shoot has been removed, and the *trans* root to the opposite one. This concept is illustrated in figure 3.3.

The first four results (tables 3.7 and 3.8) refer to the homo-graft controls for the subsequent hetero-grafts (tables 3.8 and 3.9)

Homo-grafts:

(nts382 : nts382 + nitrate)

When two nts382 plants were approach grafted in the presence of 5.5 mM KNO_3, they developed 1064 ± 429 and 1060 ± 522 nodules on their roots. The removal of one of the shoots 2 days prior to inoculation (-2D) suppressed nodulation on the *cis* (or corresponding) root by 72% (296 ± 127) compared to the intact control. Nodulation on the *trans* (opposite) root was suppressed by 29% (749 ± 104).

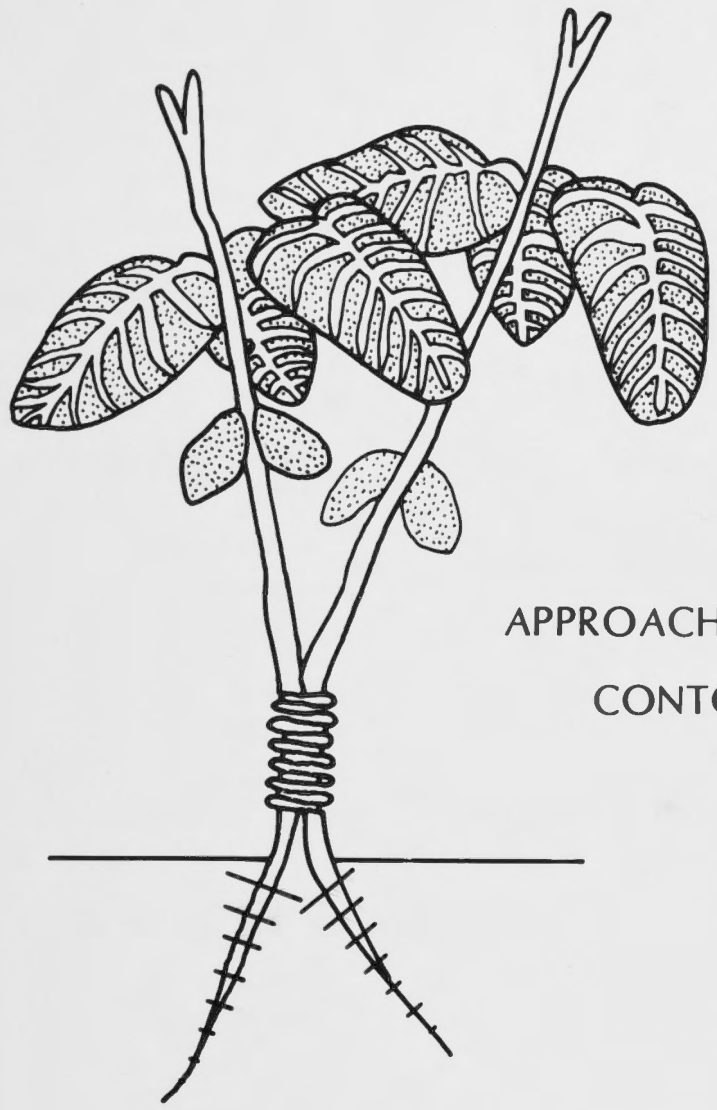
Removal of one of the nts382 shoots 2 days after inoculation (+2D) resulted in less suppression of nodulation on both sides: 414 ± 200 nodules (61 % suppression) on the *cis* root and 931 ± 255 nodules (12 % suppression) on the *trans* root.

The strong suppression of nodulation associated with the -2D removal probably occurred because the graft union had not completely sealed, and thus optimal photosynthate translocation to the roots would not be achieved and hence the nodule development would be suppressed. This is supported by root weight data which demonstrated the suppression of root weight from 0.66 ± 0.39 to 0.29 ± 0.12 g on the *cis* root and 0.65 ± 0.11 to 0.48 ± 0.30 g on the *trans* root.

By delaying the nts382 shoot removal until 2 days after inoculation the graft union had time to heal, and hence translocation of photosynthate was increased. Growth of both root systems was enhanced. The +2D *cis* root weight was 0.81 ± 0.19 g compared to 0.65 ± 0.11 g for control, and the +2D *trans* root was 0.93 ± 0.05 g compared to 0.66 ± 0.39 g control.

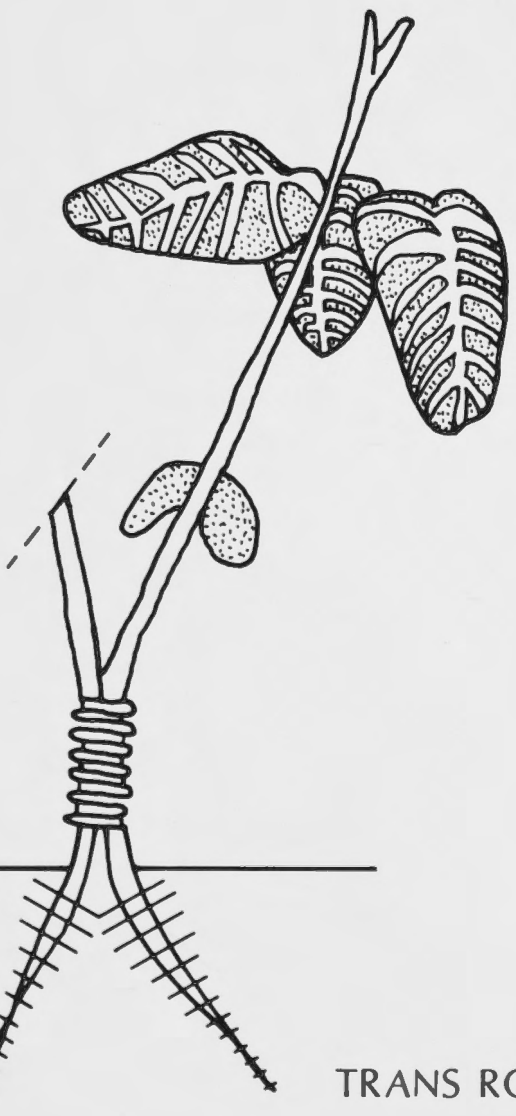
Additionally, the +2D roots had been inoculated for 48 hours prior to shoot removal, which is long enough for early infection events to occur (see Matthews, 1987; Calvert *et al.*, 1984). As such the nodulation on the +2D *cis* root was greater than for the -2D *cis* root but still less than the intact system.

Figure 3.3 **Illustration of the labelling of cis and trans roots after the removal of one shoot from an approach graft.**



APPROACH GRAFT

CONTOL



SHOOT REMOVAL

(either 2 days before

or 2 days after

inoculation)

CIS ROOT

TRANS ROOT

Table 3.7: Approach grafting of nts382 soybeans and the effect on nodulation of the subsequent removal of one shoot (Experiment 4)

GRAFT	Nodule Number	Root dry wt. (g)	Nodule Number / Plant dry wt. (g)
382 : 382 + N			
Side 1 : intact 382	1060 ± 522	0.66 ± 0.39	1334 ± 238
Side 2 : intact 382	1064 ± 429	0.65 ± 0.11	1389 ± 405
382 : 382 + N			
Side 1 : intact 382	749 ± 104	0.48 ± 0.30	1827 ± 548
Side 2 : 382 shoot removed 2 days prior to inoculation	296 ± 127	0.29 ± 0.12	1012 ± 12
382 : 382 + N			
Side 1 : intact 382	931 ± 255	0.93 ± 0.05	1158 ± 361
Side 2 : 382 shoot removed 2 days after inoculation	414 ± 200	0.81 ± 0.19	1013 ± 293
382 : 382 - N			
Side 1 : intact 382	526 ± 118	0.25 ± 0.06	1899 ± 260
Side 2 : intact 382	504 ± 70	0.35 ± 0.05	1456 ± 227
382 : 382 - N			
Side 1 : intact 382	700 ± 134	0.25 ± 0.06	2831 ± 318
Side 2 : 382 shoot removed 2 days prior to inoculation	328 ± 105	0.20 ± 0.04	1689 ± 423
382 : 382 - N			
Side 1 : intact 382	575 ± 200	0.15 ± 0.03	3915 ± 1183
Side 2 : 382 shoot removed 2 days after inoculation	161 ± 127	0.21 ± 1.00	1093 ± 180

All data expressed as $\bar{x}$ ± s.d; 382 = nts382; +N = presence of 0.5 mM KNO_3 ; - N = absence of nitrate in the nutrient solution. Number of replicates in this experiment between 5 and 6.

Table 3.8: Approach grafting of Bragg soybeans and the effect on nodulation of the subsequent removal of one shoot (Experiment 4)

GRAFT	Nodule Number	Root dry wt. (g)	Nodule Number / Plant dry wt. (g)
Bragg : Bragg + N			
Side 1 : intact Bragg	94 ± 27	1.47 ± 0.57	70 ± 23
Side 2 : intact Bragg	113 ± 13	1.12 ± 0.21	103 ± 29
Bragg : Bragg + N			
Side 1 : intact Bragg	115 ± 22	0.75 ± 0.20	161 ± 34
Side 2 : Bragg shoot removed 2 days prior to inoculation	40 ± 5	1.00 ± 0.32	44 ± 34
Bragg : Bragg + N			
Side 1 : intact Bragg	105 ± 31	0.47 ± 0.32	297 ± 144
Side 2 : Bragg shoot removed 2 days after inoculation	34 ± 14	0.88 ± 0.44	45 ± 24
Bragg: Bragg - N			
Side 1 : intact Bragg	92 ± 44	0.39 ± 0.15	299 ± 77
Side 2 : intact Bragg	117 ± 30	0.33 ± 0.03	343 ± 97
Bragg : Bragg - N			
Side 1 : intact Bragg	148 ± 68	0.39 ± 0.13	404 ± 53
Side 2 : Bragg shoot removed 2 days prior to inoculation	75 ± 8	0.36 ± 0.07	187 ± 29
Bragg : Bragg - N			
Side 1 : intact Bragg	109 ± 8	0.36 ± 0.06	309 ± 31
Side 2 : Bragg shoot removed 2 days after inoculation	34 ± 13	0.33 ± 0.20	93 ± 30

All data expressed as $\bar{x} \pm$ s.d; +N = presence of 0.5 mM KNO_3 ; - N = absence of nitrate in the nutrient solution. Number of replicates in this experiment between 4 and 6.

Table 3.9: **Approach grafting of nts382 to Bragg soybeans and the effect on nodulation of the subsequent removal of one of the shoots (Absence of nitrate) (Experiment 4)**

GRAFT	Nodule Number	Root dry wt. (g)	Nodule Number / Plant dry wt. (g)
Bragg : 382 - N			
Side 1 : intact Bragg	103 ± 68	0.26 ± 0.16	358 ± 46
Side 2 : intact 382	442 ± 158	0.22 ± 0.08	1781 ± 444
Bragg : 382 - N			
Side 1 : intact Bragg	146 ± 62	0.40 ± 0.16	401 ± 187
Side 2 : 382 shoot removed 2 days prior to inoculation	51 ± 21	0.28 ± 0.13	198 ± 129
Bragg : 382 - N			
Side 1 : intact Bragg	121 ± 73	0.17 ± 0.05	764 ± 291
Side 2 : 382 shoot removed 2 days after inoculation	17 ± 13	0.10 ± 0.04	131 ± 44
Bragg : 382 - N			
Side 1 : intact Bragg	103 ± 68	0.26 ± 0.16	358 ± 46
Side 2 : intact 382	442 ± 158	0.22 ± 0.08	1781 ± 444
Bragg : 382 - N			
Side 1 : intact 382	341 ± 152	0.15 ± 0.02	2455 ± 1412
Side 2 : Bragg shoot removed 2 days prior to inoculation	17 ± 2	0.09 ± 0.01	207 ± 42
Bragg : 382 - N			
Side 1 : intact 382	349 ± 29	0.20 ± 0.06	2493 ± 1209
Side 2 : Bragg shoot removed 2 days after inoculation	104 ± 57	0.20 ± 0.10	380 ± 154

+N = presence of 0.5 mM KNO_3 ; - N = absence of nitrate in the nutrient solution.
* all data expressed as $\bar{x}$ ± s.d. Number of replicates in this experiment between 5 and 10.

Table 3.10: **Approach grafting of nts382 to Bragg soybeans and the effect on nodulation of the subsequent removal of one of the shoots (Presence of nitrate) (Experiment 4)**

GRAFT	Nodule Number	Root dry wt. (g)	Nodule Number / Plant dry wt. (g)
Bragg : 382 + N			
Side 1: intact Bragg	106 ± 66	0.68 ± 0.40	129 ± 45
Side 2 : intact 382	401 ± 67	0.27 ± 0.15	1821 ± 707
Bragg : 382 + N			
Side 1 : intact Bragg	53 ± 35	0.25 ± 0.10	197 ± 65
Side 2 : 382 shoots removed2 days prior to inoculation	104 ± 49	0.87 ± 0.24	145 ± 49
Bragg : 382 + N			
Side 1 : intact Bragg	109 ± 43	0.93 ± 0.06	26 ± 16
Side 2 : 382 shoots removed 2 days after inoculation	31 ± 21	0.20 ± 0.04	1080 ± 526
Bragg : 382 + N			
Side 1 : intact Bragg	106 ± 66	0.68 ± 0.40	129 ± 45
Side 2 : intact 382	401 ± 67	0.27 ± 0.15	1821 ± 707
Bragg : 382 + N			
Side 1 : intact 382	246 ± 98	0.14 ± 0.03	2064 ± 499
Side 2 : Bragg shoot removed 2 days prior to inoculation	25 ± 12	0.27 ± 0.11	85 ± 14
Bragg : 382 + N			
Side 1 : intact 382	294 ± 8	0.19 ± 0.01	1590 ± 106
Side 2 : Bragg shoot removed 2 days after inoculation	329 ± 44	0.42 ± 0.06	784 ± 1

All data expressed as $\bar{x}$ ± s.d. 382 = nts382; +N = presence of 0.5 mM KNO_3 ; - N = absence of nitrate in the nutrient solution. Number of replicates in this experiment varies between 5 and 11.

Nodulation on the +2D *trans* root was higher than the -2D *trans* root and in fact recovered to the control level. The reduced nodulation on the -2D root was attributed to reduced photosynthetic potential, caused by one shoot supporting two roots.

(nts382 : nts382 minus nitrate)

In the absence of nitrate, there was normal nodulation on the *trans* root regardless or whether the *cis* shoot was removed two days before or two days after inoculation. However, this was not so on the *cis* root, where nodulation was suppressed from 504 ± 70 and 526 ± 118 on the roots of the intact system to 328 ± 105 when the shoot was removed two days before (-2D) inoculation. When the shoot was removed two days after inoculation (+2D) nodule number was reduced to 161 ± 127. Both these results were attributed to mechanical damage and reduced photosynthetic potential, although why the +2D result was so much less than the -2D result is not clear.

(Bragg : Bragg plus nitrate)

In the presence of nitrate, Bragg homo-grafts developed 94 ± 27 and 113 ± 13 nodules. The subsequent removal of one of the shoots either two days before or two days after inoculation had no effect on the *trans* root. However, nodulation on the *cis* root was suppressed equally regardless of whether the shoot was removed -2D (40 ± 5) or +2D (34 ± 15).

(Bragg : Bragg minus nitrate)

In the absence of nitrate the number of nodules which developed on the Bragg homo-graft were 92 ± 44 and 117 ± 30. The removal of one of the shoots, either -2D or + 2D, had no significant effect on the *trans* root (as seen in the plus nitrate homo-graft). However, nodule number on the *cis* root was suppressed to 75 ± 8 when the shoot was removed at -2D. When the shoot removal was delayed to +2D nodule number was further reduced to 34 ±13. This shows the same trend as the nts382 : nts382 minus nitrate result.

Hetero-grafts

(Bragg : nts382 minus nitrate)

When Bragg was grafted to nts382 in the absence of nitrate, the number of nodules which formed on the Bragg root were 103 ± 68 as compared to 117 ± 30 on a Bragg homo-graft, thus demonstrating no significant difference. Nts382 roots developed 442 ± 158 nodules as compared to 526 ± 118 on a nts382 homo-graft. Whilst the nodulation of nts382 was slightly suppressed the difference was not significant.

Nodule number differences in the hetero-grafts were not observed in the minus nitrate grafts because the absence of nitrate raised the threshold level for autoregulation. That is, in the absence of nitrate, the Bragg needs to produce more nodules (to provide ammonia

for the plant) and so does not autoregulated to the same degree as grafts grown in the presence of sufficient nitrate.

(Bragg : nts382 minus nitrate) nts382 removal

The removal of the nts382 shoot two days prior to inoculation significantly suppressed *cis* nodulation from 442 ± 158 to 51 ± 21 units, and Bragg nodule number was unafffected. Nodulation on the nts382 root was further suppressed to 17 ±13 units when the *cis* shoot was removed two days after inoculation, however Bragg nodule number remained unaffected.

These results indicate that nts382 nodulation was initially suppressed due to the limitation of assimilate. It is possible that by +2D the Bragg shoot was signalling the nts382 roots to autoregulate, thus accounting for the suppression of nts382 nodulation.

(Bragg : nts382 minus nitrate) Bragg removal

The removal of the Bragg shoot two days prior to inoculation resulted in a significant reduction in the nodulation of that root from 103 ± 68 to 17 ± 2 units. This was accompanied by a corresponding reduction in root weight from 0.26 ± 0.16 g. per plant to 0.09 ± 0.01 g.per plant. This indicates that nodule suppression was attributable to reduced photosynthate deprivation. Nts382 showed a slight *trans* effect with nodule number being reduced from 442 ± 158 to 341 ± 158, however the reduction was not significant.

When the removal of the Bragg shoot was delayed until two days after inoculation, nodule number on the Bragg root recovered to 104 ± 57 (intact level), whilst nts382 remained at the -2D level (349 ± 29). These results indicate that in the absence of nitrate, the supposed autoregulation signal in Bragg is not elicited.

(Bragg : nts382 plus nitrate)

When Bragg was grafted to nts382 in the presence of nitrate, 106 ± 66 and 401 ± 67 nodules developed on the respective roots. There was no significant difference in Bragg nodulation compared to the Bragg homo-graft (113 ± 13). However, nts382 nodulation was suppressed by 62% relative to the 382 homo-graft level (1064 ± 429). This is a slightly different result to that observed in experiment 1, in which Bragg nodulation increased and nts382 nodulation decreased. However in this experiment, plants were grown in the presence of 0.5 mM KNO_3, and in experiment 1 they were grown in the presence of 5.5 mM KNO_3. However, the data is still indicative of a dilution of the Bragg autoregulation signal between the two plants.

(Bragg : nts382 plus nitrate) nts382 removal

When the nts382 shoot was removed two days prior to inoculation, nodule number per plant on the *trans* root (Bragg) was suppressed to 53 ± 35, and 104 ± 49 on the *cis* root. However when removal of the 382 shoot was delayed until two days after inoculation, Bragg nodule number remained at the intact level. Nodule number on the nts382 root did not recover and was further suppressed to 31 ± 21. Surprisingly however, the nts382 root weight was enhanced, so the suppression was not due to an inadequate translocation of photosynthate. The explanation for this is not totally clear. The data may indicate that the decapitated nts382 root was under the control of the intact Bragg shoot, and hence succumbed to a Bragg autoregulation signal, or alternatively, that by removing the nts382 shoot, one also removed the source of nodule enhancing signal. This would also account for the reduction in nts382 nodulation and the absence of change on the Bragg root.

(Bragg : nts382 plus nitrate) Bragg removal

The removal of the Bragg shoot demonstrated more interesting results. When removed two days prior to inoculation, Bragg nodulation was reduced from 106 ± 66 to 25 ± 12, and root weight was suppressed from 0.68 ± 0.40 g. per plant to 0.27 ± 0.11 g.per plant. This was a result of structural resource (possibly photosynthate) depletion. However, when the removal of the Bragg shoot was delayed until two days after inoculation, Bragg nodulation was significantly increased from 106 ± 66 to 329 ± 44 nodules per root side constituting a 3 fold increase in nodulation compared to that on the Bragg root of the intact control. The effect on nts382 nodule number of removing the Bragg shoot, either two days before or after inoculation was to suppress it from 401 ± 67 to 246 ± 98 and 294 ± 44 respectively.

3.6.4 DISCUSSION

Thus for nts382 : Bragg hetero-grafts grown in the presence of 0.5 mM KNO_3, the removal of the Bragg shoot two days after inoculation resulted in an increase in Bragg nodulation and a reduction in nts382 nodulation. It is still not clear whether the removal of the Bragg shoot resulted in the loss of the source of an autoregulatory signal, since the data could also be explained by the action of an nts382 nodule enhancement signal being diluted between the two plants. This would hold true if the nodule enhancement signal was suppressed or masked by the presence of the intact Bragg shoot.

This is more clearly demonstrated when we look at homo-grafts compared to hetero-grafts in which one shoot is removed two days after inoculation:

(A) Side 1 (Bragg intact): 105 ± 31 nodules
Side 2 (Bragg removed): 34 ± 14 nodules

(B) Side 1 (nts382 intact): 294 ± 8 nodules
Side 2 (Bragg removed): 329 ± 44 nodules

i.e. when the Bragg shoot is removed, and the nts382 shoot is left intact (B) Bragg nodule number is 10 fold higher than if a Bragg shoot is left intact (A). Additionally, the presence of a single nts382 shoot results in a total nodule number on the hetero-graft of 623. However when a single Bragg shoot is left on a homo-graft, the total nodule count is 139.

(C) Side 1 (nts382 intact): 931 ± 255 nodules
Side 2 (nts382 removed): 414 ± 200 nodules

(D) Side 1 (Bragg intact): 109 ± 43 nodules
Side 2 (nts382 removed): 31 ± 21 nodules

i.e. When the nts382 shoot is removed, and the Bragg shoot is left intact (D), nts382 nodule number was 13 fold less than if a nts382 shoot was left intact (C). Additionally, when a single Bragg shoot was left on the hetero-graft, the total nodule count was 140. However, when a single nts382 shoot was left on the homo-graft, the total nodule count was 1345.

These results confirm that the control of nodule number is systemically regulated, and in hetero-grafts can be controlled by either a single Bragg or single nts382 shoot. However, it is still unclear whether the Bragg shoot is the source of autoregulation signal or whether nts382 shoots are the source of a nodule enhancement signal.

3.7 EXPERIMENT 5: EFFECT ON NODULATION OF WEDGE GRAFTING EITHER CHALLENGED OR UN-CHALLENGED SHOOTS ONTO UN-CHALLENGED ROOTS

3.7.1 INTRODUCTION

Following the approach graft experiments, the question arose whether the suppression of wild-type nodulation is caused by an inhibitor in the shoots of Bragg, the absence or reduction of this signal in the super-nodulating mutant, or the presence of a nodule enhancement signal which is permanently switched on in nts382. The previous shoot removal experiment did not answer this, but did show that in approach grafts the removal of either a Bragg or nts382 shoot resulted in the remaining shoot controlling the nodule phenotype.

To investigate this problem, a series of wedge grafting experiments were set up, the first using Bragg and the second using the super-nodulating mutant. Shoots from challenged plants (i.e. shoots from plants which had been grown intact in the presence of *B. japonicum* strain USDA 110 for either 10, 12, 14 or 18 days) were grafted onto un-challenged (or un-inoculated) roots, and then subsequently inoculated (5 days after grafting). The control experiment involved grafting un-challenged shoots of comparable age onto un-challenged roots with a subsequent inoculation five days later. The protocol for experiment 4 is shown overleaf and in figure 3.4.

3.7.2 Design

Day 0 : Plant seeds
Water daily with 0.5 mM KNO_3 in 1/4 strength Herridge nutrient solution

Day 10 : Divide plant population into two,
Inoculate half with *B. japonicum* USDA110 (these are now "challenged").
Leave the rest un-inoculated (un-challenged).

continued

Experiment	Control
Day 10 : Graft inoculated (challenged) shoot onto un-inoculated (un-challenged) root	Day 10 : Graft un-inoculated (un-challenged) shoot onto un-inoculated (un-challenged) root
Leave under mist spray for 5 days Inoculate with USDA 110	Leave under mist spray for 5 days Inoculate with USDA110
Day 12) Repeat as for Day 14 *) day 10 Day 18)	Day 12) Repeat as for Day 14 *) day 10 Day 18)
Day 38 Harvest	Day 38 Harvest

* Begin watering with full strength Herridge nutrient solution.

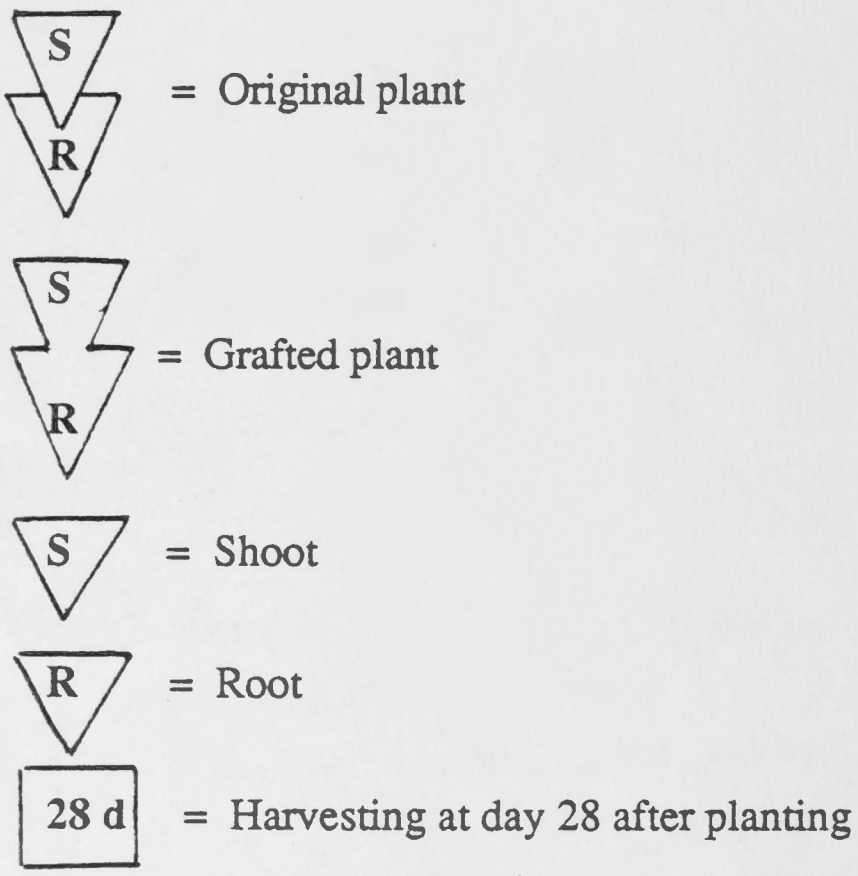
3.7.3 RESULTS : Wedge grafts using challenged and un-challenged shoots

Figure 3.5 shows that the longer a Bragg plant remains intact in the presence of *B. japonicum*, prior to its shoot being grafted onto an un-inoculated (or un-challenged) root, the less nodules were able to form. Table 3.11 shows that 43 ± 12 nodules formed when the Bragg shoot had been challenged for 10 days, 35 ± 14 when challenged for 12 days, 30 ± 9 when challenged for 14 days and 8 ± 3 nodules formed when the challenge period was increased to 17 days.

The control experiment, in which progressively older, un-inoculated (un-challenged) shoots were grafted onto un-inoculated roots, (then subsequently inoculated), showed no suppression of nodulation, i.e. shoots with 10 days of pre-graft, un-challenged growth developed 45 ± 12 nodules. Those with 12,14 and 17 days of pre-graft growth developed 43 ± 13, 47 ± 8 and 50 ± 12 nodules respectively (see table 3.11 and figure 3.5).

In the parallel part of the experiment using nts382 plants (table 3.12) no significant difference was observed in the resultant nodule number for grafts in which the shoot

Figure 3.4 Experimental lay-out for the challenge experiment (Experiment 5)



Experimental Lay-out for the Challenge Experiment

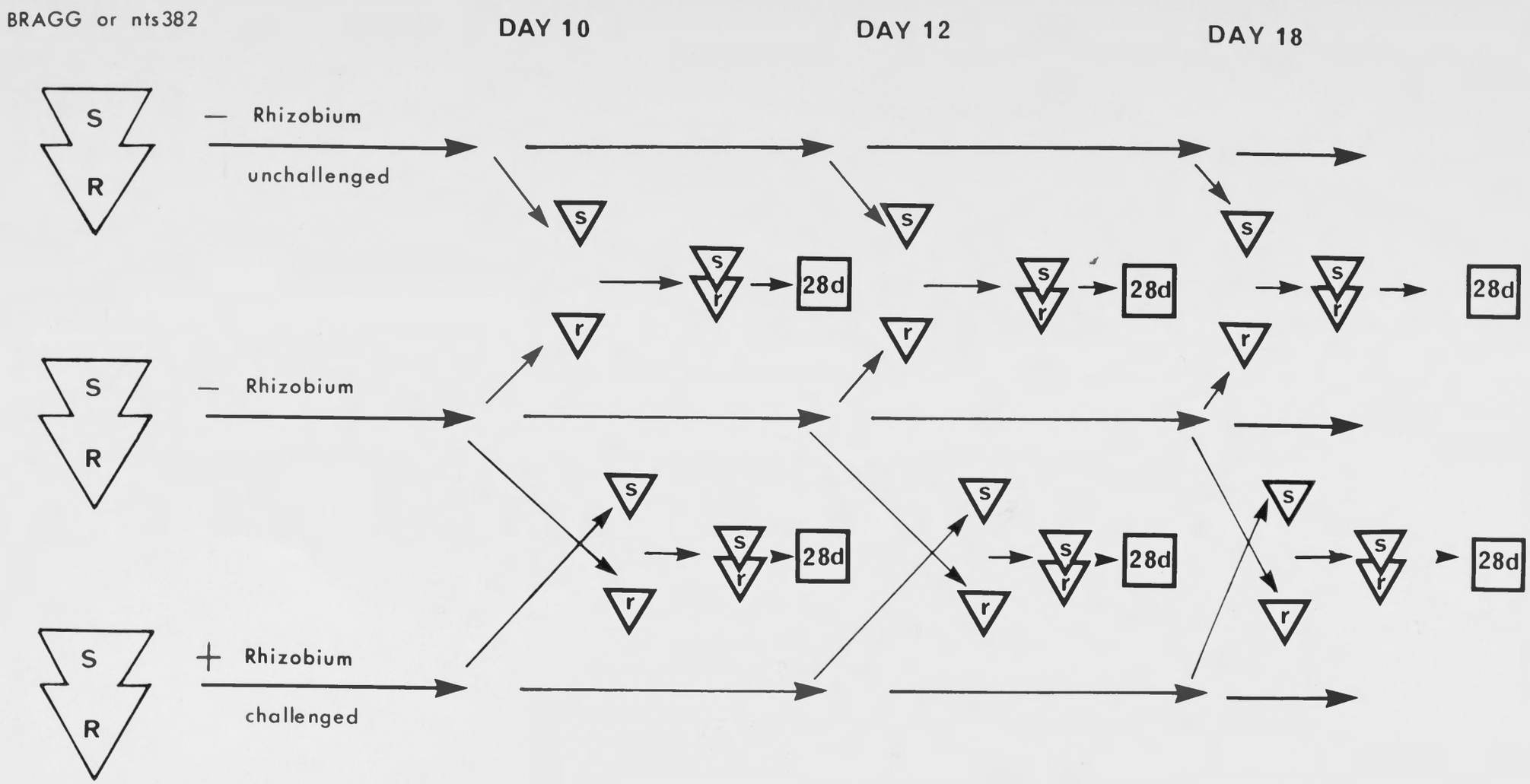
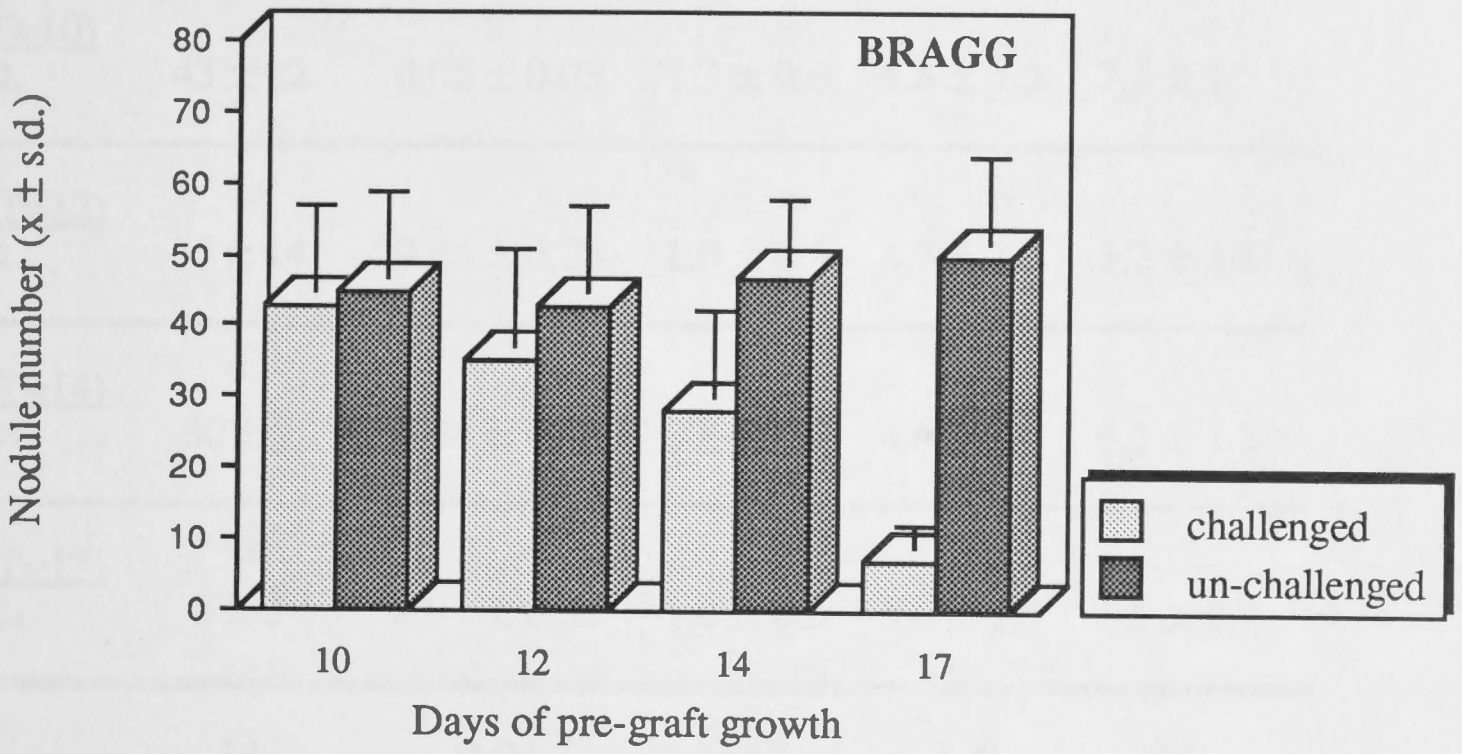


Figure 3.5 **Histogram of nodule number (mean ± s.d) on roots of challenged and un-challenged Bragg and nts382 plants**

Challenged plants are those whose shoots derived from inoculated plants,

Un-challenged plants are those whose shoots derived from un-inoculated plants.


BRAGG
Nodule number (x ± s.d.)
80
70
60
50
40
30
20
10
0
10
12
14
17
Days of pre-graft growth
challenged
un-challenged

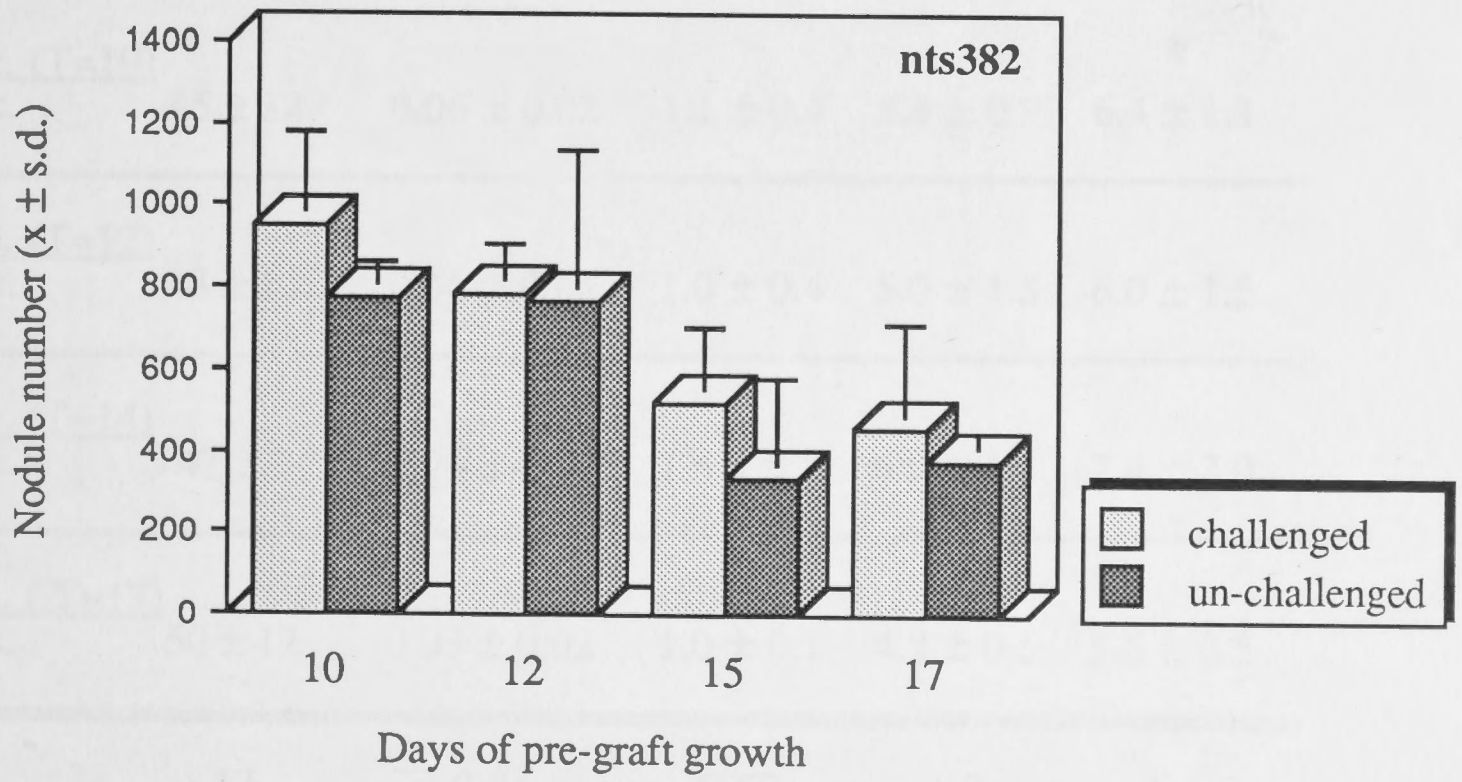

nts382
Nodule number (x ± s.d.)
1400
1200
1000
800
600
400
200
0
10
12
15
17
Days of pre-graft growth
challenged
un-challenged

Table 3.11: Effect of grafting either challenged or un-challenged soybean shoots onto un-inoculated roots on the subsequent nodulation and plant growth of Bragg plants (Experiment 5)

Graft	Nodule No.	Nodule dry wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Total dry wt.(g)
B. Inoc.(T=10) B. Uninoc.	43 ± 12	0.05 ± 0.03	1.3 ± 0.6	5.8 ± 1.2	7.3 ± 1.7
B. Inoc. (T=12) B. Uninoc.	35 ± 14	0.05 ± 0.03	1.0 ± 0.5	4.7 ± 1.2	5.7 ± 1.6
B. Inoc. (T=14) B. Uninoc.	30 ± 9	0.04 ± 0.02	0.8 ± 0.4	4.4 ± 1.4	6.5 ± 1.3
B. Inoc. (T=17) B. Uninoc.	8 ± 3	0.04 ± 0.02	1.0 ± 0.2	5.0 ± 1.5	5.5 ± 2.7
LSD 0.05	14	0.04	0.65	1.6	2.0
B. Uninoc. (T=10) B. Uninoc.	45 ± 12	0.06 ± 0.02	1.1 ± 0.4	5.4 ± 0.9	6.4 ± 1.1
B. Uninoc. (T=12) B. Uninoc.	43 ± 13	0.06 ± 0.04	1.0 ± 0.4	5.0 ± 1.5	6.0 ± 1.5
B. Uninoc. (T=14) B. Uninoc.	47 ± 8	0.06 ± 0.03	1.6 ± 0.8	6.0 ± 1.5	7.4 ± 2.0
B. Uninoc. (T=17) B. Uninoc.	50 ± 12	0.03 ± 0.02	1.0 ± 0.1	4.8 ± 0.5	5.6 ± 0.5
LSD 0.05	17	0.04	0.50	1.3	1.5

All data expressed as $\bar{x}$ ± s.d; B = Bragg; inoc. = inoculated; uninoc. = un-inoculated; T = days of pre-graft growth. Number of replicates between 4 and 5.

Table 3.12: Effect of grafting either challenged or un-challenged soybean shoots onto un-inoculated roots on the subsequent nodulation and plant growth of nts382 plants. (Experiment 5)

Graft	Nodule No.	Nodule dry wt (g)	Root dry wt (g)	Shoot dry wt (g)	Total dry wt (g)
382 Inoc.(T=10)					
382 Uninoc.	951 ± 193	0.43 ± 0.04	0.4 ± 0.1	2.7 ± 0.3	3.4 ± 0.5
382 Inoc. (T=12)					
382 Uninoc	783 ± 87	0.29 ± 0.09	0.5 ± 0.2	2.8 ± 0.4	3.5 ± 0.5
382 Inoc. (T=15)					
382 Uninoc.	519 ± 149	0.17 ± 0.06	0.3 ± 0.1	1.7 ± 0.7	2.1 ± 0.8
382 Inoc. (T=17)					
382 Uninoc.	455 ± 223	0.04 ± 0.02	0.3 ± 0.2	0.9 ± 0.4	1.2 ± 0.5
LSD 0.05	331	0.09	0.28	0.7	0.9
382 Uninoc. (T=10)					
382 Uninoc.	772 ± 58	0.24 ± 0.05	0.3 ± 0.1	2.0 ± 0.5	2.5 ± 0.6
382 Uninoc. (T=12)					
382 Uninoc	763 ± 335	0.29 ± 0.28	0.5 ± 0.2	2.4 ± 0.8	3.2 ± 1.4
382 Uninoc. (T=15)					
382 Uninoc.	331 ± 218	0.16 ± 0.05	0.4 ± 0.1	1.8 ± 0.4	2.3 ± 0.4
382 Uninoc. (T=17)					
382 Uninoc.	381 ± 37	0.08 ± 0.03	0.3 ± 0.1	1.5 ± 0.3	1.8 ± 0.4
LSD 0.05	297	0.17	0.2	0.8	1.0

All data is expressed as $\bar{x} \pm$ s.d; 382 = nts382; inoc. = inoculated; uninoc. = un-inoculated; T = days of pre-graft growth. Number of replicates between 4 and 5.

derived from either a challenged or an un-challenged plant of the same age. This indicated that nts382 had no significant autoregulation mechanism. The nts382 shoot which had been challenged for 10 days developed 951 ± 193 nodules and its un-challenged control developed 772 ± 58. When the graft was left for 12 days, those grafts with challenged shoots developed 783 ± 87 nodules and those with un-challenged shoots developed 763 ± 335 nodules. The nts382 shoot which had been challenged for 15 days developed 519 ± 149 nodules and its un-challenged control developed 331 ± 218 nodules. Finally, grafts whose shoots had been challenged for 17 days developed 455 ± 223 nodules whilst the un-challenged control developed 381 ± 37 nodules. In short, there was no difference between challenged and un-challenged shoots. However, there was a progressive decrease in plant nodule number for both challenged and un-challenged shoots over time. This was caused by the decreasing period during which nodules were allowed to develop prior to harvest. Day *et al.* (1986) showed that nts382 nearly doubles its nodules per week. In contrast, Bragg, because of its autoregulation maintains nodule number after an initial "burst." Thus the nts382 data potentially requires to be handicapped, but this was not felt necessary for the interpretation of this effect.

The above results indicate that the autoregulation factor is present as an inhibitory signal in the shoots of Bragg plants and is initiated as a result of a inoculation. The nts382 mutant be altered in has a greatly reduced capacity to elicit the shoot signal both in the presence and absence of nitrate.

The nitrate tolerance of the nts382 mutant may be due to the absence of the shoot inhibitor, whose activity in wild-type is (a) increased by inoculation and (b) further potentiated in the root through nitrate. How it works though is not known.

The results from the challenged set of Bragg grafts provide information about the feed-back mechanism of nodulation control. When a 10 day challenged Bragg shoot is grafted onto and un-challenged (un-inoculated) Bragg root and the whole graft is subsequently inoculated, there is no differences in the resultant nodulation as compared to those grafts in which an un-challenged (but similar age) Bragg shoot is used. However, when Bragg plants are grown in the presence of *Brady-rhizobium* for 12, 14 or 17 days prior to grafting onto un-challenged roots (then subsequently inoculated) there are progressivley fewer nodules formed. The reduction of nodule number to 8 ± 3 nodules shows strong suppression of nodulation, and indicates that newly inoculated, un-nodulated roots do not have the ability to initiate a feed-forward mechanism of nodule control. That is, it cannot produce a signal to over-ride the shoot derived autoregulation signal. Additionally, it is possible that once the root derived infection signals reach the shoot, they bring about changes in the shoot physiology such that the autoregulation signal is elicited. These

changes must be long lasting since the autoregulation signal may be synthesized and translocated to root tissue which is only poorly nodulated.

3.8 CONCLUSIONS

The results from experiments in chapter 3 provide the following conclusions:

1. Nitrate stimulates the suppression of nodulation on Bragg plants,

2. Nts382 demonstrates tolerance to nitrate suppression of nodulation,

3. Bragg : nts382 approach grafts grown in the absence of nitrate demonstrated no change in nodule number compared to their respective homo-graft controls,

4. Bragg : nts382 approach grafts grown in the presence of nitrate demonstrated significant increases in Bragg, and significant decreases in nts382 nodule number,

5. The changes in (4) were caused by nitrate eliciting an autoregulation signal in Bragg shoots which was shoot derived, systemically translocated, and diluted between the two plants, and was capable of suppressing nodulation on nts382,

6. Nitrate had no effect on the amount of translocation indicator (^{14}C 2,4-D) recovered from the roots of Bragg : nts382 approach grafts as compared to those grafts grown in the absence of nitrate. (i.e. the absence of nodule number alteration in (3) was not caused by the inability of graft junctions to heal in the absence of nitrate, thus preventing translocation of an autoregulation factor),

7. Bragg plants grown in the absence of nitrate produce a basal level of autoregulation factor in response to inoculation by *Brady-rhizobium*, but the signal is stimulated in the presence of nitrate, and results in suppression of nodulation,

8. Nts382 and nts1116 mutants demonstrated no ability to elicit the autoregulation response, either in the presence of inoculum or nitrate.

CHAPTER FOUR

EFFECT OF APPLIED GROWTH REGULATORS ON NODULATION AND PLANT GROWTH OF SUPER-NODULATING AND WILD-TYPE SOYBEANS

4.1 INTRODUCTION

Members of the *Rhizobeaceae*, such as *Agrobacterium, Rhizobium* and *Bradyrhizobium,* interact with plants to produce biological niches for the continued survival of the bacterium. In all cases plant development is altered. For example, *Agrobacterium* produces a tumour on the transformed roots, and *Bradyrhizobium* induces root (or stem as in the case of infection of *Sesbania, Neptunia* and *Aeschynomenae* spp. by *Rhizobium*) nodules.

Such ontogenic changes in plant differentiation are certain to involve changes in plant hormones (growth regulators). As the control of soybean (cv. Bragg) nodulation was shown during the course of this study to be shoot derived (Delves *et al.* 1986; Chapter 3), and since hormones are defined as biological signal molecules which (a) act at low concentration and (b) act at a site different to their synthesis, it was thought appropriate to expand this study to include a baseline investigation of the effects of applied hormones on nodulation in both the wild-type and super-nodulation soybeans.

There is considerable published evidence which demonstrates that changes in endogenous hormone levels occur when plants become infected by micro-organisms (Puppo and Rigaud 1978; Williams and de Mallorca 1982; Badenoch-Jones *et al.* 1983). Similarly, exogenous application of plant growth regulators may mimic symptoms caused by symbiotic or pathogenic interactions. These plant developmental changes may be the result of secretion of hormones by micro-organisms or may be due to a plant response to infection.

This chapter is concerned with understanding the involvement of growth regulators in the control of nodule initiation and development. The nitrate tolerant, nodule autoregulation, soybean mutant nts382 was used in comparison with its wild-type parent cultivar Bragg to investigate the involvement of, and responses to, the application of the phyto-hormones, indole acetic acid, the gibberellic acids (GA_3 and GA_4), abscisic acid and a gibberellic acid biosynthesis inhibitor chloro-choline-chloride (CCC) in the autoregulation phenomenon.

The nts382 mutant was chosen, as past studies (Carroll *et al.* 1985; Day *et al.* 1986) had shown a variety of growth related differences compared to wild-type plants (grown under identical conditions) which were indicative of an alteration in the endogenous phyto-hormone balance.

Further indications that hormones or growth regulators are involved in autoregulation of nodulation come from wedge grafting studies by Delves *et al.* (1986), who showed that when nts382 shoots were grafted onto un-inoculated Bragg roots, and subsequently

inoculated, the resultant phenotype was super-nodulated. Conversely, when Bragg shoots were grafted onto nts382 roots, the nodule phenotype was wild-type (see table 3.1). This demonstrated that nodulation was shoot controlled and the autoregulation factor was systemically translocated. Results from approach graft and re-feeding experiments outlined in chapter 3 further demonstrated that a systemic factor was involved in the inhibition of nodulation.

4.1.1 GENERAL HORMONE ACTION IN PLANTS

A hormone is an organic substance which, acting in small quantities, and being produced in any one part of the organism, is transferred to another part and there influences a specific physiological process presumably through a reaction involving its attachment to some stereo-specific site (Leopold and Nooden 1984).

There are five known major classes of hormones involved in the regulation of plant developmental processes. Early studies designated specific functions to each class of hormones; auxin was first described as the regulator of cell enlargement, gibberellic acid as the regulator of overall stem growth, cytokinin as the regulator of cell division, abscisic acid as the regulator of abscission and dormancy and ethylene as the regulator of fruit ripening (Leopold and Nooden 1984).

However, it is now known that this single hormone control concept has shortcomings since it has been found that none of the hormones have exclusive control over any phase of growth or development.

Since hormones are synthesized in one location and translocated to a target site, it must be noted that the extraction and identification of plant hormones from various plant tissues does not automatically mean that the compound is serving a regulatory function. Another complication in the involvement of plant hormones is that the compound which is synthesized and translocated may not be the same one acting at the target site. For example, environmental stress experienced by roots (due to water-logging) is found to cause wilting of the leaves; the roots synthesize ACC (1-amino-cyclo-propane - 1-carboxylic acid) and this chemical is transported to the leaves where it is converted to ethylene which is the active principle causing the wilting response (Bradford and Yang 1980). Therefore, using the traditional concept of a hormone as a translocated chemical messenger, ACC may be more aptly considered to be a hormone than ethylene.

For a target tissue to respond in a particular manner, a hormone must bind to a receptor site (which may or may not be a protein). Hall and Thomas (1987) suggested that when the hormone binds to the receptor, the shape of the receptor changes which in turn

changes the properties of the receptor protein. This permits either the direct initiation of some process or an interaction between the receptor and some other component that leads to a particular biochemical response such as activation of a gene and synthesis of a particular enzyme.

Four general types on interaction may commonly occur between hormones:

(a) Ratios between hormones, e.g. the ratio of cytokinins to auxins in regeneration of plant cells in tissue culture changes from high during bud differentiation to low during root regeneration (see Vardjan and Nitsch 1961; Gresshoff 1976 and numerous other examples);

(b) Opposing actions between pairs of hormones, e.g. auxin and cytokinin in apical dominance (Wickson and Thimann 1958); ethylene and auxin with the former stimulating leaf abscission and the latter inhibiting it (Hall 1952);

(c) One hormone may alter the concentration of another by modifying hormone biosynthesis, translocation or inactivation; (Trevawas 1982);

(d) Sequential regulation by several hormones, e.g. oat colyoptile tissue has been shown to pass through a stage in which growth was stimulated by gibberellin, followed by a stage of cytokinin stimulation with the final growth phase controlled by auxin (Wright 1961).

4.1.2 LOCATION OF THE BIOSYNTHESIS OF HORMONES AND THEIR MODES OF ACTION

Auxins are organic substances which at low concentrations (< 1 mM) promote growth along the longitudinal axis, and inhibit elongation of the roots, when applied to shoots of plants. Auxin is not transported directly in the vascular tissue but instead appears to be transported in cells associated with the phloem. This transport system moves the hormone basipetally from the natural auxin sources in the shoot to the root to regulate growth and other developmental processes.

In studies with auxins, the growth increment increases with increasing auxin concentration up to a maximum. A plateau follows and, after this higher concentrations result in a decrease in elongation. This optimum may vary with plant species, pH, buffer and ion concentration. A hormone can have different activities in different tissues. Essentially this means that an auxin can be inhibitory as well as stimulatory depending on the target tissues, its physiological state (i.e. sensitivity) and concentration. Clearly, in view of the receptor hypothesis there must be a correlation between receptor density,

Figure 4.1 **Structures of the plant growth substances discussed in chapter 4 experiments**

IAA = Indole acetic acid,

ABA = Abscisic acid,

GA_3 = Gibberellic acid 3,

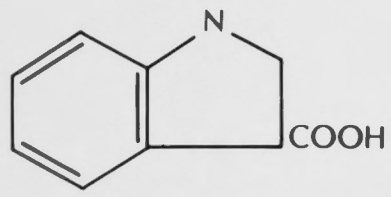
GA_4 = Gibberellic acid 4,

CCC = Chloro-choline chloride,

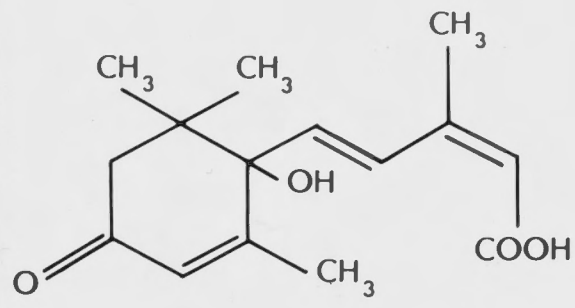
Kinetin,

Gibbane Carbon Skeleton

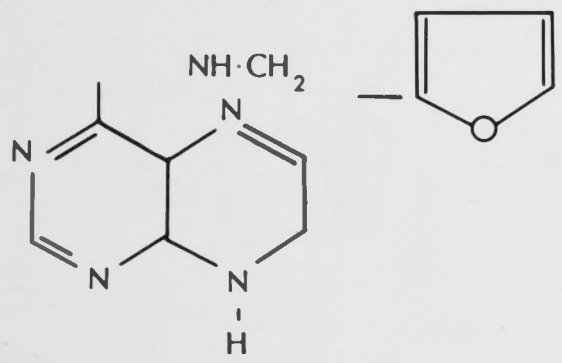
IAA



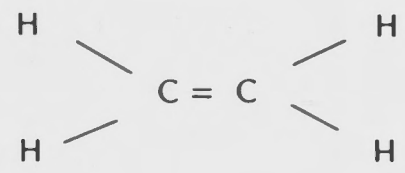
ABA



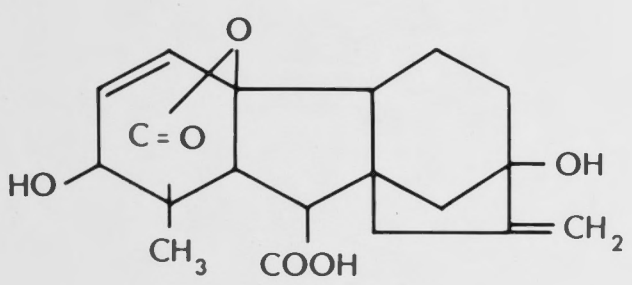
KINETIN



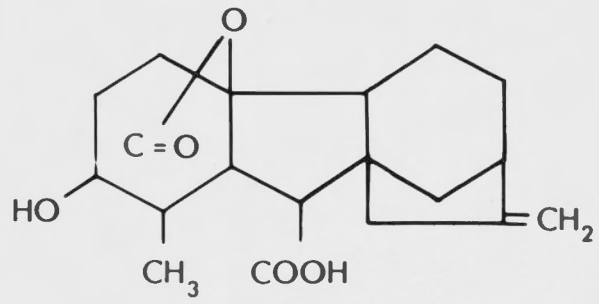
ETHYLENE



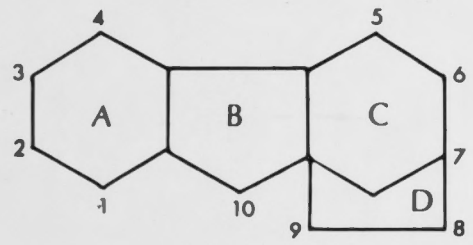
GA_3



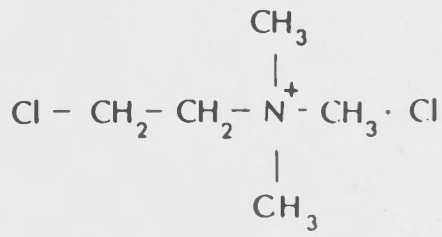
GA_4



GIBBANE CARBON SKELETON



C C C



hormone concentration and level of saturation. If only a few receptors are present, the saturation will occur early (i.e. at low concentration) and the same biological effect may be achieved. The nature of these relations has not yet been determined, and so leads to obvious difficulties in studying hormone physiology.

Investigations of the metabolism of endogenous auxin have concentrated on the origin and fate of IAA in plant tissues, since it is considered the principal auxin (Wareing and Phillips 1975).

It is widely accepted that the main sites of auxin biosynthesis are the meristematic tissues and young growing parts of plants, especially the shoot apex, buds and developing seeds. IAA biosynthesis has also been demonstrated to occur in plant tissue culture (e.g. soybean cotyledon callus; Black and Hamilton 1976).

Many plant growth responses result from the interaction of auxin with other hormones. For example while cytokinins and auxins participate in the imposition of apical dominance - auxin in the shoot and cytokinins in the root, in the absence of each other they promote branching in the opposite portion of the root (Scott 1984).

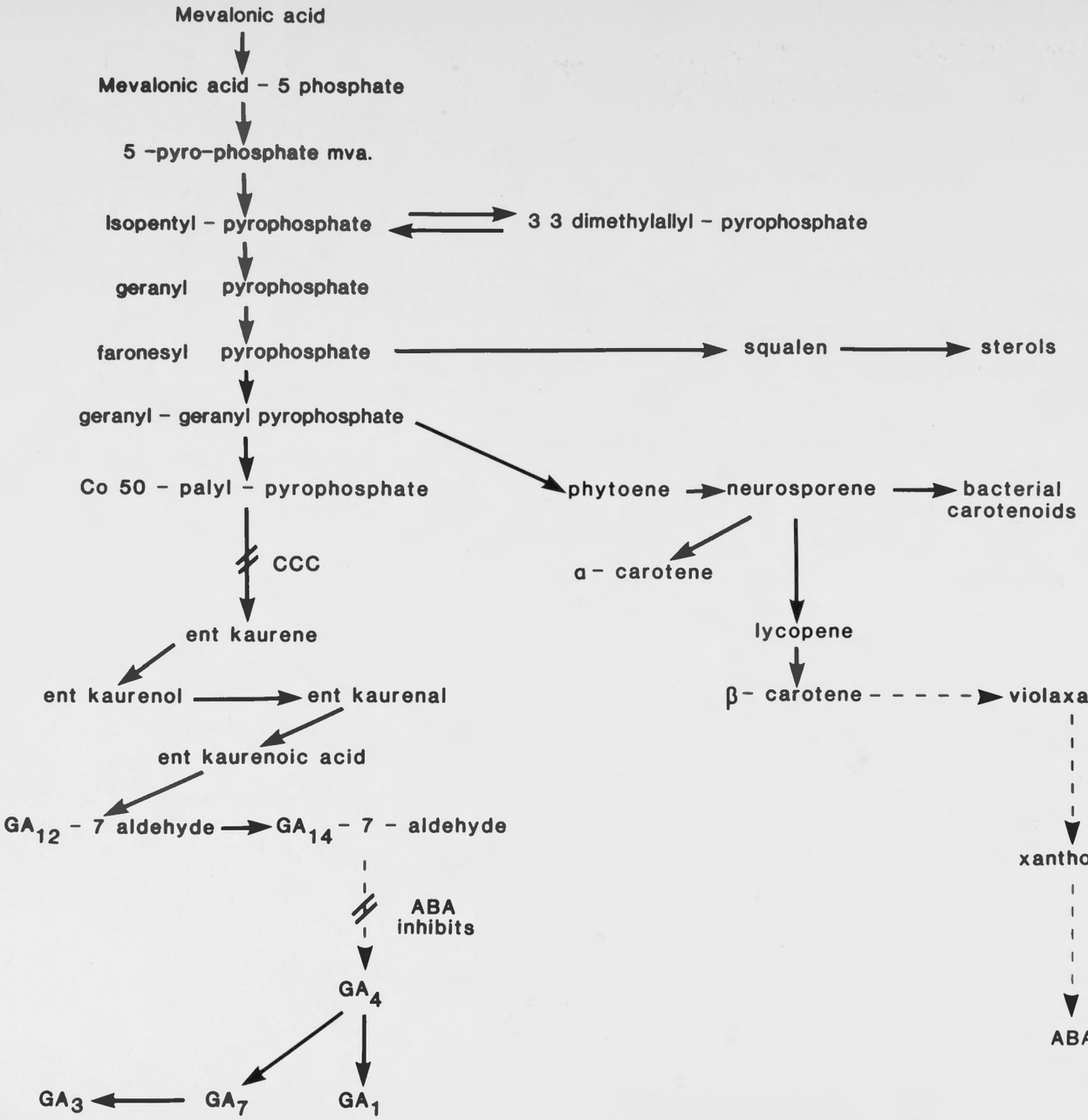
A **gibberellin** is a compound which is active in gibberellin bioassays and possesses a gibbane ring skeleton. When gibberellic acid (GA_3) was applied to many species of intact growing plants it induced abnormally great extension of stems and leaves, but the response was always greater when genetic dwarfs of various plant species were used (Potts *et al.* 1983). To date 72 gibberellins have been discovered which produce effects similar to that elicited by GA_3 (see MacMillan 1985; Graebe 1985). These compounds have been designated gibberellin A_1, A_2, A_3, A_4 etc. and have the same basic structure (the gibbane carbon skeleton, see fig. 4.1) as GA_3 but differ from one another in the number and position of substituent groups of the ring system and the number and the degree of saturation in the "A" ring (see fig. 4.1). Results from a number of investigations conclude that the shoot is a primary site of gibberellic acid biosynthesis in vegetative parts of peas and other di-cotyledenous plants (Lockhart 1957). Figure 4.2 shows the current view on the synthesis of gibberellins from mevalonic acid (see also Graebe 1985).

Cytokinins in general are substituted adenines, although di-phenyl urea is claimed to have cytokinin activity (see figure 4.1). Cytokinins were originally regarded as being particularly important in the stimulation of cell division (hence the name) and differentiation in plant tissue culture but more recently have been implicated in various other physiological processes such as senescence and apical dominance. They also promote seed germination and induce nutrient flow to cytokinin treated areas, or to those

Figure 4.2 The pathway of mevalonic acid to gibberellic acid and abscisic acid, showing the location of inhibition by CCC and ABA (after Neill *et al.* 1984; Graebe 1985; Zeevaart *et al.* 1985)

- - - - represents the indirect pathway of ABA synthesis proposed by Neill *et al.* 1984 and Zeevaart *et al.* 1985,

N.B. The conversion of GA_4 to GA_3 has been established for *Gibberella fujikuroi* (Fletcher *et al.* 1958), but as yet the intermediacy of GA_4 in higher plants has not yet been established.


Mevalonic acid
Mevalonic acid – 5 phosphate
5 –pyro–phosphate mva.
Isopentyl – pyrophosphate
3 3 dimethylallyl – pyrophosphate
geranyl pyrophosphate
faronesyl pyrophosphate
squalen
sterols
geranyl – geranyl pyrophosphate
Co 50 – palyl – pyrophosphate
phytoene
neurosporene
bacterial carotenoids
CCC
α – carotene
ent kaurene
lycopene
ent kaurenol
ent kaurenal
β – carotene
violaxanthin
ent kaurenoic acid
xanthoxin
GA_{12} – 7 aldehyde
GA_{14} – 7 – aldehyde
ABA inhibits
ABA
GA_4
GA_3
GA_7
GA_1

parts of the plant in which cytokinins are synthesized (Wilkins 1979). However the mechanism(s) for this are unknown. Cytokinins are predominately synthesized in the quiescent centre found in root tips.

Abscisic acid (see figure 4.1) plays an important role in leaf abscision and the regulation of bud dormancy, and is probably also involved in the regulation of a number of growth processes. ABA also plays a role in mediating responses to water stress. For example, application of ABA to turgid leaves causes rapid closure of stomata and levels of ABA in leaves of mesophytic plants increase many times under conditions of water stress (Zeevaart *et al.* 1985).

Early reports have indicated that ABA is directly synthesized from mevalonic acid (see references in Zeevaart *et al.*1985). That is, a C_{15} precursor, presumably farenesyl pyrophosphate, is cyclisized and then converted to ABA. However, there are now lines of evidence that favour the indirect pathway in which ABA is synthesized by the degradation of xanthophylls (C_{40}) (see Neill *et al.* 1984; Zeevaart *et al.* 1985; and fig. 4.2).

Ethylene exists as a gas at normal temperatures and therefore acts as a gaseous hormone. The particular responses which involve ethylene are mainly those which are known to occur in the presence of relatively high auxin concentrations. Some of the known effects of ethylene are:

(i) induction of epinasty, (ii) inhibition of elongation growth in stems and roots of most species, (iii) promotion of leaf abscission, (iv) inhibiton of the basipetal polar and lateral transport of auxin, (v) regulation of the feedback mechanism of the level of endogenous auxin (i.e. a high concentration of auxin stimulates ethylene formation, but the ethylene then in some way causes a lowering of the auxin level in the tissue; see Wareing and Phillips 1975).

Much of the information incorporated into hormonal control models is based on the data from two experimental approaches, namely (1) the application of plant hormones, the so called "spray and pray" approach and (2) the measurement of changes in endogenous hormone levels.by use of bioassays, gas chromatography, High Pressure Liquid Chromatography (HPLC) and more recently GC-Mass spectroscopy (Leopold and Nooden 1984) (see section 4.1.4).

The external application of hormones is a logical and inexpensive experimental method which is based on the premise that if a growth process is significantly modified by an applied substance, a regulatory role is implied for that hormone. However, the

uncertainty in these experiments lies firstly in the fact that applied hormones can sometimes cause responses which are not apparently regulated by that hormone in normal development. For example, non specific application can result in secondary effects such as the closure of stomata by guard cell action, which then affects photosynthesis, and subsequently acetylene reduction.

Secondly, while the applied concentration of hormone may be known, the amount of substance absorbed by the plant is unknown. Consequently, micro-injections of various concentrations of hormones provide a better method of application.

4.1.3 A GENERAL SURVEY OF THE EFFECTS OF APPLICATIONS OF HORMONES ON NODULATION

When investigating the hormone effects on morphogenesis, thought must not only be given to the argument of sensitivity (see above) but also the balance between synthesis, translocation to the target site, and inactivation (i.e. metabolism). Also, hormones can be autoregulatory, in as much as a hormones's synthesis can regulate further synthesis. One such example is that auxin stimulates its own path of translocation (Sachs 1975)

Hormones should also be viewed as components of morphogenic dimensions. Not only does a plant exist in an up versus down relationship, (shoot and root) but also within the radial and tissue distribution. There are sources (sites of synthesis or secretion) and sinks (sites of metabolism). Such morphogenic fields control the differentiation of plant tissues, e.g. stomata or vascular cambium (Warren Wilson and Warren Wilson 1984). Thus, hormonal effects on nodulation may not only be primary, i.e. affecting cell types directly, but also secondary, i.e. affecting morphogenic gradients and fields. Such mechanisms would result in the potential transmission of a "signal" without an actual molecule moving (similar to an electric current, or a wave in the sea). These thoughts are of value when considering hormonal effects on nodulation which (as is shown below) often involve "signalling" across several cell distances (e.g. cortical cell induction, autoregulation or induction of a variable O_2 barrier within the nodule).

In an attempt to mimic the *in vivo* initiation and development of nodules, plant hormones and growth regulators have been applied to the leaves and roots of a variety of species (Letham *et al.* 1978). The following is a summary of the effects on nodulation and associated plant growth, demonstrated as the result of the application of various growth regulators

4.1.3.1 Gibberellic acid

Thurber *et al.* (1958) used a daily foliar application of GA_3 (50 ppm) which greatly reduced nodulation, decreased plant dry weight, and enhanced elongation and lateral bud growth of *Phaseolus vulgaris*. Similarly, Mes (1959) sprayed *Vicia villosa* plants twice weekly with 10 ppm GA_3 and noticed a two third reduction in nodulation, the decrease being dependant on the age of the plants at which the acid was applied.

Similar experiments were done by Fletcher *et al.* (1958) who noted that two applications of 50 ppm GA (10 days apart) had no adverse effect on the nodulation of white clover (*Trifolium repens*). However, when gibberellin was included in the growing medium it inhibited nodulation of *Trifolium subterraneum* (Kefford *et al.* 1960) and also of excised roots of *P. vulgaris* (at 1 ppm).

This investigation was continued by Williams and de Mallorca (1984) who examined the effect of exogenous applications of gibberellins and the growth retardant *B*-chloroethyl-trimethyl-ammonium-chloride (CCC, see 4.1.3.5) on root nodule formation and activity in *Glycine soya* cv Jupiter. They showed that daily foliar applications of GA_3 (2.89×10^{-6} M) delayed the formation of nodule initials and reduced the numbers, nodule mass and specific nitrogenase activity of nodules by 43 %, 31 % and 47 % respectively, without affecting plant growth. Similar effects on nodulation were produced by foliar applications of GA_4 (3.01×10^{-5} M) or GA_7 (2.89×10^{-6} M) or by the addition of GA_3 (6.30×10^{-5} M) to the rooting medium.

The disparity in these nodulation and plant growth responses discussed above were most likely due to differences in plant species and experimental conditions such as (i) age of plant when GA was applied, (ii) frequency of application, (iii) mode of application of hormone, (iv) hormone concentration, (v) day length and light intensity during the experimental period. Despite the variation in responses, the data implicates the involvement of gibberellins in the regulation mechanism of nodulation and suggests that the endogenous root content of gibberellic acids may have an (auto)regulatory role in both the infection process and the subsequent development of nodules.

4.1.3.2 Abscisic acid (ABA)

Exogenous ABA application has been identified as a strong inhibitor of nodulation. Phillips (1971) showed that 1.9×10^{-6} M ABA, applied to the roots of *Pisum sativum L.* cv. Alaska (peas), reduced the number of nodules per plant 61 % without affecting root or shoot growth and appeared to act by inhibiting the cortical cell divisions required for root nodule formation. Bano and Hillman (1986) applied ABA (10^{-8} M m^{-3}) to the root systems of 45 - 54 day old *Faba vulgaris* plants via hydroponics culture. They

observed that nodule primordia receiving ABA were arrested and small ineffective, brownish coloured nodules were formed. Early degeneration of bacteroid tissue was seen in developing nodules. Developed nodules receiving ABA became elongated, developed a corky texture and fragile vascular connections at their point of attachment to the host root. Durley *et al.* (1976) showed that ABA inhibited hypocotyl elongation and root growth and also inhibited the conversion of GA_4 to GA_1.

4.1.3.3 Indole acetic acid (IAA)

High concentrations of IAA (10^{-3} M) markedly reduced root elongation and the nodule number of excised roots of *Phaseolus vulgaris* . The treated roots were shorter and thicker than the controls but similar in total fresh weight (Cartwright 1967). It was suggested that the effects on nodulation were probably secondary to those of elongation of the roots which probably controlled the number of sites available for nodule formation.

Other work investigating the effect of IAA on nodulation comes from Valera and Alexander (1965) who showed that IAA (10^{-8} M) could reverse the nitrate inhibition of nodulation in lucerne. Similarly, Munns (1968 a,b) showed that the addition of IAA to solution cultures of *Medicgo sativa* alleviated but did not eliminate the inhibition of nodulation by acidity and nitrate. IAA increased nodule number only if it was present during the development of infection threads.

4.1.3.4 Ethylene

The first report on the inhibitory effect of ethylene on nodulation of legumes by *Rhizobium* was presented by Small *et al.* (1968) during the study of the effect of environmental factors on nodule formation of *P. vulgaris* roots. This effect was later confirmed by Grobelaar *et al.* (1971), Drennan and Norton (1972) and Goodlass and Smith (1979). More recently Ligero *et al.* (1987) showed that inoculation led to an increase in ethylene biosynthesis. Of interest in relation to this observation is the recent description of increased phytoalexin and hydroxyproline rich protein synthesis in response to ethylene (Ecker and Davis 1987). However, two responses need to be distinguished, namely the hyper-sensitive response to parasitic infections, and the response by the host to a potential symbiont. It has been shown that coumestrol, an isoflavone, is strongly increased after actinorhizal infection, but is not greatly increased after host specific infections such as in the *Bradyrhizobium* - soybean system. This indicates that the plant can distinguish between specific (i.e. symbiotic) and non-specific (i.e. parasitic) infections. Thus, in soybean infections, increased ethylene biosynthesis and phytoalexin concentration (e.g. isoflavones) may not necessarily occur.

4.1.3.5 Cytokinins

It has been suggested that the initiation of cell divisions in the mature root cortex is under hormonal control (Phillips and Torrey 1970) since (a) Das *et al.* (1956) showed that cytokinins and auxins were required for cell division in tobacco parenchyma; (b) Matthysse and Torrey (1967) showed that polyploid cells in the cortex of pea root segments required cytokinin for mitotic activity; (c) *in vitro* studies of pea root cortical systems revealed that exogenous auxins and cytokinins could initiate patterns of endo-reduplication and cell enlargements similar to those which occurred in nodule development (Torrey and Barrios 1969; Libbenga *et al.* 1973); and (d) Phillips and Torrey (1970) showed that cytokinin production of *Rhizobium japonicum* strain 61A68 *in vitro* could be used to promote cortical cell proliferation in host tissue. Also, Bauer *et al* (1985) showed that sub-epidermal cell division is soybean could be induced by exogenous application of benzyladenine, suggesting that the bacterium produces such cell division substances.

4.1.3.6 Chloro-choline-chloride

Chloro-choline-chloride, also known as CCC, chloremquat, and 2-chloro-ethyl-trimethyl-ammonium-chloride, is a quaternary ammonium plant growth retardant with the structure as shown in figure 4.1. Early work showed that CCC blocks gibberellin biosynthesis in the fungus *Fusarium moniliform* and in higher plants and, that the feeding of CCC to cultures of *Gibberella* sp. caused enrichment of geranyl-geranyl-pyrophosphate (Harada and Lang 1965). Experiments by Dennis *et al.*(1965) indicate that CCC inhibits the enzyme kaurene synthetase, and that CCC changes the proportion of free to bound forms of gibberellins.

Williams and de Mallorca (1984) examined the effect of this growth retardant (6.30×10^{-5} M) on nodulation of pouch grown *Glycine soya* cv. jupiter and demonstrated an increase in nodule number by 56 %.

These observations indicate that a cell division factor is limiting within the host plant tissue and it has been postulated that it may be provided by the bacterial symbiont since it has been demonstrated that *in vitro* culture of *Rhizobium* produce auxins (Badenoch-Jones *et al.* 1982a,b; Dullaart 1967; Sinha and Basu 1981; Wang *et al.* 1982) and cytokinins (Phillips and Torrey 1970; and Syono and Torrey 1976). However whether these *Rhizobium* derived hormones in fact play a direct role in the initiation of soybean nodulation is not known.

4.1.4 TECHNIQUES USED IN THE MEASUREMENT OF CHANGES IN ENDOGENOUS LEVELS OF GROWTH HORMONES.

Bioassays were the original method of detection but have fallen into disfavour since they are generally subject to interference from other compounds in the preparations being tested and are less specific than other methods.

Gas chromatography (GC) has been used to measure putative hormone peaks in eluent profiles from gas chromatographs, however resolution of peaks may require further fractionation as often multiple molecular species are found within one peak. GC analysis also requires that most hormones be derivetized, via a somewhat hazardous technique, thus further complicating the technique.

High Pressure Liquid Chromatography (HPLC) analysis also has problems since the relative non-specificity of most detectors and low resolving power (compared with capillary GC) questions the identity of peaks in the HPLC eluent profile.

GC-Mass spectroscopy allows the verification of the identity as well as the quantification of the hormone and as such is the method of choice. However, the high cost, labour intensity and low availability of this system puts it out of the reach of the majority of investigators (Leopold and Nooden 1984).

The HPLC technique has been used by Dr A.J. Krotzky of the Botany department (ANU), for studies surrounding the fluctuations of phyto-hormones as a response to inoculation and nitrate application in soybean cv. Bragg and nts382. Reference will be made to work done by his group as well as to collaborative studies done on this particular project.

4.1.5 PAST STUDIES OF ENDOGENOUS LEVELS OF NODULE PHYTO-HORMONES

The amounts of extractable growth regulators such as auxins (Thimann 1936; Pate 1958; Dullaart 1967; Charbonneau and Newcomb 1985; Badenoch-Jones *et al.* 1983); cytokinins (Henson and Wheeler 1976; Syono and Torrey 1976; Newcomb *et al.* 1977); abscisic acid (Williams and de Mallorca 1982; Charbonneau and Newcomb 1985) and gibberellins (Dullaart and Duba 1970; Radley 1961) are reported to be greater in *Rhizobium* induced nodules than in other regions of the root.

During nodule senescence the level of growth promoting chemicals, particularly IAA, decreased following an increase in the endogenous level of growth retarding chemicals (Libbenga and Bogers 1974). Watts *et al.* (1983) also showed that endogenous levels of

abscisic acid in the dormant root nodules of *Alnus glutinosa* was 2.5 times higher than of actively growing nodules.

4.1.6 HORMONE SUSCEPTIBILITY ARGUMENT

The molecular basis of sensitivity of plant tissues to growth substances is not known, but it is thought to be dependent upon the presence of specific (receptor) proteins in particular tissues. Trewavas (1981) states that the limiting factor in the growth substance response is the effective concentration of the growth substance-receptor complex. The equation:

Growth substance + receptor $\rightleftharpoons$ growth substance - receptor

indicates that this concentration may be varied either by changing the growth substance concentration or by varying the level of tissue receptor, i.e. altering sensitivity.

This level may be varied by environmental stimuli such as light, water, mineral nutrition and developmental age of the tissue. The nutritional status of the plant, particularly its supply of nitrogen, has a significant effect on root derived cytokinins and consequently on shoot physiology and development. For example, leaves of *Nicotiana rustica* plants responded to kinetin when the roots were deprived of nitrate, but not when the nutrient solution contained nitrate (Goodwin *et al.* 1978)

According to Sinnott (1960), well nourished plants have a greater number of cells which on average are larger than those of the mal-nourished equivalent. Trewavas (1982) also showed that conditions which resulted in poor growth reduced the rate of protein synthesis, and increased protein degradation. As receptors are likely to be proteins they will vary in this manner also. He stated that if the receptor can be equated with receptors for division and receptors for extension then well nourished plants should remain dividing for longer periods of time. The net result would also be more and longer cells.

This chapter investigates the effects on nodulation and plant growth of varying the presumptive concentration of growth substance-receptor complex, via the addition of nitrate to the rooting solution and either foliar or inter-cotyledonary injections of phyto-hormones and the growth inhibitor CCC. In particular, this study was designed to see whether a shoot applied hormone could suppress (perhaps by mimicking the missing shoot substance), the nts382 phenotype.

EXPERIMENTAL DESIGNS, RESULTS AND DISCUSSIONS

4.2 EXPERIMENT 6: EFFECT OF DAILY FOLIAR APPLICATIONS OF (A) GA_3 AND (B) CCC ON NODULATION AND PLANT GROWTH

4.2.1 INTRODUCTION

Previous studies by Thurber *et al.* (1958); Fletcher *et al.* (1958); Mes (1959); Kefford *et al.* (1960); and Williams and de Mallorca (1984) demonstrated the involvement of GA_3 in the suppression of wild-type nodulation, and Williams and de Mallorca (1984) demonstrate that CCC (a gibberellic acid biosynthesis inhibitor) caused an increase in wild-type nodule number (see section 4.1.3.1). This experiment was designed to investigate the role of GA_3 in wild-type nodule suppression, and to examine its effect on the autoregulation mutant nts382.

4.2.2 DESIGN - EXPERIMENTS 1(A) AND (B)

Seeds of *Glycine max.* wild type cultivar Bragg and its super-nodulation mutant nts382 were planted in 20 cm pots of sterile sand / vermiculite (3:1) at a density of 10 plants per pot. Ten days after sowing the seedlings were inoculated with *B.japonicum* strain USDA 110 (10^9 viable cells per pot) from pre-inoculated peat bags (see section 2.4.2). Plants were watered daily with Herridge nutrient solution (see table 2.1) immediately prior to treatment with growth regulators.

The treatments used in this experiment involved spraying a daily foliar application of either ;

(a) GA_3 (2.89×10^{-6} M), or
(b) CCC (6.30×10^{-5} M), or
(c) neither,

onto Bragg or nts382 plants grown in either the presence (5.5 mM KNO_3) or absence of nitrate in the nutrient solution. Concentrations of GA_3 and CCC were as suggested by Williams and de Mallorca (1984). Spraying was begun immediately after inoculation and was continued for 28 days, at which time the plants were harvested and nodules were detached and counted. Roots, shoots and nodules were separated and dried at 65° C for 48 hours prior to weighing.

4.2.3 RESULTS: EXPERIMENT 6(A) - EFFECTS OF FOLIAR APPLICATIONS OF GA_3 ON NODULATION AND PLANT GROWTH

Bragg minus nitrate plus GA_3

Tables 4.1 and 4.2 show that Bragg plants grown in the absence of nitrate developed 187 ± 33 nodules. Similar plants treated with a daily foliar application of GA_3 (2.89 x 10^{-6} M) showed a slight reduction in nodule number (159 ± 40), however the difference was not significant. Similarly, there was no change in specific nodule weight. Thus GA_3 spray resulted in slightly fewer nodules, but of the same size as the control. The GA_3 treatment increased both the shoot and root dry weights per plant (to 138 % and 184 % of the controls respectively, see appendix 1) resulting in an increase in total plant dry weight. This indicates that GA_3 applied to non-nitrate treated Bragg signalled the plant to direct photosynthate to roots and shoots without affecting nodulation.

Bragg plus nitrate plus GA_3

Bragg plants grown in the presence of 5.5 mM KNO_3 showed nitrate inhibition of nodulation, resulting in 22 ± 6 nodules per plant. The application of GA_3 to similar plants caused a slight (but not significant) suppression of nodule number to 15 ± 6. Nodule dry weight was slightly reduced from 0.05 ± 0.02 g. per plant to 0.03 ± 0.03 g. per plant caused by a significant suppression of specific nodule dry weight to 48 % of control plants (see table 4.2 and appendix 2). Root dry weight was significantly suppressed to 57 % of control weights (see appendix 1), but shoot and total dry weights were not suppressed. Thus in the presence of nitrate, GA_3 reduced Bragg root development and nodule size, but otherwise left the wild-type plant un-affected.

Nts382 minus nitrate plus GA_3

Daily foliar applications of GA_3 significantly reduced the number of nodules which formed on nts382 plants grown in the absence of nitrate to 67 % of control plants (i.e. from 587 ± 55 nodules to 391 ± 83, see table 4.1 and appendix 1), but had no effect on the total nodule weight per plant. Specific nodule weight was increased compared to control plants, indicating nodule compensation (i.e. fewer, but bigger, nodules). Both root and shoot dry weights were significantly increased as a result of the GA_3 sprays. A significant reduction in nodule number was observed when nodule number was expressed per gram of plant dry weight (table 4.2). Therefore, in the absence of nitrate.

Nts382 plus nitrate plus GA_3

Nts382 plants grown in the presence of nitrate (5.5 mM KNO_3) and treated with GA_3 demonstrated a pronounced suppression of nodule number (from 459 ± 88 to 183 ± 85) and total nodule weight per plant (table 4.1). Specific nodule weight was unaffected

Table 4.1 Effect of daily foliar applications of GA_3 and CCC on nodulation and plant growth (Experiment 6)

Treatment	N	Nodule Number	Nodule Dry wt. (g)	Root Dry wt. (g)
nts 382 + N	9	459 ± 88^a	0.25 ± 0.07^a	0.55 ± 0.13^a
nts 382 + N + CCC	8	776 ± 128^b	0.33 ± 0.08^b	0.65 ± 0.13^a
nts 382 + N + GA_3	8	183 ± 85^c	0.10 ± 0.07^c	0.36 ± 0.08^b
LSD 0.05		103	0.07	0.12
B + N	7	22 ± 6^a	0.05 ± 0.03^a	0.74 ± 0.31^a
B + N + CCC	8	82 ± 19^b	0.10 ± 0.03^b	1.18 ± 0.48^b
B + N + GA_3	8	15 ± 6^a	0.03 ± 0.03^a	0.42 ± 0.13^c
LSD 0.05		13	0.03	0.34
nts 382 - N	6	587 ± 55^a	0.27 ± 0.06^a	0.15 ± 0.03^a
nts 382 - N + CCC	8	1016 ± 287^b	0.34 ± 0.11^a	0.33 ± 0.06^b
nts 382 - N + GA_3	8	391 ± 83^c	0.26 ± 0.06^a	0.25 ± 0.04^b
LSD 0.05		97	0.10	0.05
B - N	7	187 ± 33^a	0.17 ± 0.06^a	0.25 ± 0.16^a
B - N + CCC	5	256 ± 39^b	0.20 ± 0.09^a	0.48 ± 0.10^b
B - N - GA_3	5	159 ± 40^a	0.18 ± 0.04^a	0.46 ± 0.07^b
LSD 0.05		46	0.08	0.11

All data expressed ax $\bar{x} \pm$ S.D; +N = Herridge solution supplemented with 5.5 mm KNO_3; -N Herridge solution minus nitrate; CCC = chloro chlorine chloride (6.30×10^{-5} M); GA_3 = gibberellic acid (2.89×10^{-6} M);
Numbers in the same grouping of three, with different alphabetic superscripts are significantlydifferent as determined by the LSD 0.05. N= Number of replicates

Table 4.2 Effect of daily foliar applications of GA_3 and CCC on nodulation and plant growth. (Experiment 6)

Treatment	N	Total Dry wt. (g)	Specific nodule wt. (g)	Nodule No. / g. total plant dry wt.
nts 382 + N	9	5.04 ± 0.56^a	0.5 ± 0.2^a	95 ± 10^a
nts 382 + N + CCC	8	6.05 ± 0.81^b	0.5 ± 0.1^a	130 ± 32^b
nts 382 + N + GA_3	8	4.50 ± 0.70^a	0.6 ± 0.2^a	40 ± 17^c
LSD 0.05		0.70	0.2	23
B + N	7	6.15 ± 1.26^a	2.9 ± 1.8^a	4 ± 1^a
B + N + CCC	8	10.75 ± 2.21^b	1.3 ± 0.6^b	10 ± 4^b
B + N + GA_3	8	4.54 ± 0.77^a	1.4 ± 0.8^b	4 ± 2^a
LSD 0.05		1.55	1.3	2
nts 382 - N	6	2.05 ± 0.48^a	0.5 ± 0.1^a	296 ± 57^a
nts 382 - N + CCC	8	2.89 ± 0.48^b	0.4 ± 0.1^a	358 ± 141^a
nts 382 - N + GA_3	8	2.60 ± 0.27^b	0.7 ± 0.2^a	151 ± 33^b
LSD 0.05		0.39	0.2	93
B - N	7	2.89 ± 0.61^a	1.0 ± 0.4^a	67 ± 25^a
B - N + CCC	5	2.91 ± 0.69^a	0.8 ± 0.3^a	94 ± 35^b
B - N -GA_3	5	3.53 ± 0.21^a	1.0 ± 0.2^a	25 ± 13^a
LSD 0.05		0.70	0.4	25

All data expressed as $\bar{x} \pm$ S.D; +N = Herridge solution supplemented with 5.5 mm KNO_3; - N Herridge solution minus nitrate; CCC = chloro chlorine chloride (6.30×10^{-5} M); GA_3 = gibberellic acid (2.89×10^{-6} M);
Numbers in the same grouping of three, with different alphabetic superscripts are significantlydifferent as determined by the LSD 0.05. N = Number of replicates

(table 4.2), indicating an absence of nodule weight compensation. Root dry weight was also significantly suppressed to 65 %, but shoot dry weight was not affected. Overall GA_3 did not have a significant effect on total plant dry weight. When nodule number was expressed per gram of root, shoot or total dry weight (appendix 2) significant decreases were observed (50 %, 41 % and 42 % respectively) showing that nodule number suppression was caused by suppression of plant growth.

4.2.4 RESULTS: EXPERIMENT 6(B) : EFFECT OF DAILY FOLIAR APPLICATIONS OF CCC ON NODULATION AND PLANT GROWTH

Bragg minus nitrate + CCC

When Bragg plants, grown in the absence of nitrate, were sprayed with a gibberellic acid synthesis inhibitor, CCC (table 4.1), there was a significant increase in nodule number per plant from 187 ± 33 to 256 ± 39 nodules (table 4.1) or to 137 % of control levels (appendix 1). Specific nodule weight tended towards a reduction, resulting in a steady total nodule weight. The root dry weight was significantly increased to 192 % of control plants (appendix 1). However, when nodule number was expressed per gram of total plant dry weight (table 4.2) there was a significant increase compared to control plants (94 ± 35 compared to 67 ± 25 nodules respectively).

Bragg plus nitrate + CCC

The addition of CCC to nitrate treated Bragg plants resulted in a significant increase in nodule number (373 %), nodule dry weight (200 %), root dry weight (159 %), shoot dry weight (147 %) and total plant dry weight (175 %) (appendix 1). Specific nodule dry weight however, was significantly reduced to 45 % of control plants (appendix 2) due to compensation for the increase nodule number.

When nodule number was expressed per gram of total plant dry weight (appendix 2) there was a significant increase of 278 %. Thus CCC has a greater effect on Bragg if plants are grown in the presence of nitrate. Note also that gibberellic acid effects were also dependent on nitrate.

Nts382 minus nitrate + CCC

Nts382 plants grown in the absence of nitrate responded to CCC treatment by specifically increasing nodule number (173%), however, while total nodule dry weight increased slightly, the difference was not significant from control (nts382 minus nitrate) plants. Similarly there was no significant alteration in specific nodule dry weight. Plant growth was enhanced by the application of CCC with increases in root dry weight (220

%), shoot dry weight (133 %) and total plant dry weight (131%) (table 4.2 and appendix 1).

When nodule number was expressed per gram of nodule weight it increased to 148 % but when expressed as a function of root dry weight reduced to 75 % (see appendix 2). Nodule number did not change significantly when expressed as a percentage of shoot or total dry weight (appendix 2). This suggests that the shoot was less affected than the root or total plant dry weight, which further indicates that CCC has a root effect which is reflected in nodule number, but not shoot growth. Also, this is a reversal of the usual nts382 results, where more nts382 nodules mean smaller shoots.

Nts382 plus nitrate + CCC

The application of CCC to nitrate grown mutant plants caused a significant increase in nodule number (169 %) (similar to that seen in the absence of nitrate, of 173 %) and nodule dry weight. In contrast to the minus nitrate data, there was no significant difference in root dry weight upon GA_3 treatment (118 % of control), nor was there any significant difference in shoot dry weight (122 % of control), thus indicating that the increase in nodule number was not caused by changes in root or shoot weights (see appendix 1). This is confirmed by data in appendix 2 which shows that significant increases in nodule number were noted when nodule number was expressed per gram of root (163 %), shoot (142 %) and total dry weight (137 %).

4.2.5 DISCUSSION : EFFECT OF GA_3 AND CCC SPRAY ON NODULATION AND PLANT GROWTH

These experiments showed that Bragg and nts382 responded to nitrate as anticipated from published results, i.e. nts382 was nitrate tolerant and Bragg demonstrated nitrate inhibition of nodulation. Further, both Bragg and nts382 grew better with nitrate. Secondly it was demonstrated that 2.89×10^{-6} M GA_3 (as a spray) had little effect on nodule number in Bragg; whether or not nitrate was present. In both cases there was a small hint of a trend towards a reduction in nodulation, but this was not statistically significant. This is in contrast to results from Williams and de Mallorca (1984), who showed suppression of nodulation in nitrate free grown *Glycine soya*.. This disparity could be due to the the difference in soybean cultivar used.

The application of GA_3 to Bragg plants grown in the presence of nitrate, while demonstrating no suppression of nodulation, demonstrated a significant suppression in specific nodule dry weight. This demonstrates that in the presence of nitrate GA_3 interfers with the development of nodules, such that they are smaller than normal.

In contrast, nts382 plants grown on nitrate, or grown fully symbiotically showed considerable decreases in the number of nodules per plant. The decrease was more pronounced in the presence of nitrate. These nodulation changes occured without a concommitant decrease of total plant dry weight, (if anything, GA_3 stimulated plant growth in the absence of nitrate, but inhibited slightly in the presence), resulting in pronounced changes of the specific nodule number (i.e. nodule number per gram plant dry weight).

Specific nodule weight (i.e. nodule dry weight per gram nodule number), being a measure of nodule size and thus nodule growth (rather than initiation) remained relatively constant on nts382 plants grown in the presence and absence of nitrate, and also on Bragg plants grown in the absence of nitrate except in Bragg plus high nitrate, where GA_3 treatment resulted in a halving of that parameter.

In contrast, the gibberellic acid synthesis inhibitor, CCC, significantly increased the nodule number of both Bragg and nts382 plants grown in the presence and absence of nitrate. CCC increased nodule number of nitrate grown plants more than those grown under nitrate free conditions, whereas nts382 nodulation was equally increased. This indicates that gibberellic acid is involved either directly or indirectly in the regulation of nodule number, since direct application of GA_3 as well as the presumed interference with GA biosynthesis by CCC results in changes in nodule number. (N.B. It must be noted here that in these experiments it is assumed that CCC is acting to inhibit the synthesis of gibberellic acid as previously shown by various groups (see Harada and Lang 1963; Dennis *et al.* 1965). The scope of this study did not include the biochemical verification of lowered GA pools or biosynthesis after CCC addition).

One possible explanation for the above results is that in the wild type plant, nodulation control is linked to the availability of gibberellins. Thus an autoregulated plant would perhaps be synthesizing more gibberellins than a "freely" nodulating plant. The further addition of gibberellins to an autoregulated plant then would have no further suppressive effect. However nts382 plants, being autoregulation mutants would have a pool of gibberellins too low to trigger autoregulation, compared to wild type plants and would therefore continue to nodulate freely. The application of GA_3 to these plants would trigger the autoregulation response causing a suppression of nodulation. Conversely, the application of CCC to Bragg and nts382 would have its effect on increasing nodulation under all conditions simply by reducing gibberellins in the plant.

The fact that nts382 plants were susceptible to nodule suppression by GA_3 whilst Bragg plants were not indicates that the nts382 mutation may affect a physiological / ontogenetic cascade involving the gibberellic acid pathway.

Nts382 plants grown in the presence or absence of nitrate showed no significant difference in specific nodule dry weight, and hence no compensation for the reduction in nodule tissue caused by GA_3 treatment. Similarly, CCC treatment had no effect on specific nodule weight. Thus the CCC induced increase in nodule dry weight was the result of the initiation of many more fully developed nodules.

However, while CCC increased nodule weight in Bragg grown in the presence of nitrate, it was achieved by a different method. Bragg specific nodule weight was significantly reduced indicating that the increase in nodule dry weight was the result of the initiation of many nodules which failed to develop to normal size.

Thus by reducing the synthesis of gibberellic acid (through the use of CCC) both Bragg and nts382 were signalled to initiate further infections. However, the CCC treatment exerted no control over nodule development since there was no change in specific nodule weight.

It is possible that the existing pools of GA in the Bragg plant acted as a signal in the autoregulation pathway. Thus if gibberellins are involved in the wild type autoregulation system, then the initiation of nodules would initiate an increase in the synthesis of gibberellins which would signal the suppression of further cell division.

The availability of nitrate affected the action of GA_3 in the development of root tissue. Both Bragg and nts382, in the absence of nitrate, responded to GA_3 treatment by significantly increasing root dry weight, but in the presence of nitrate, responded by significantly reducing root weights as compared to non GA_3 treated plants. This indicates that plants grown in the absence of nitrate are gibberellic acid deficient, whilst those grown in the presence of nitrate have sufficient amounts, such that additional GA_3 inhibits nodulation.

Root dry weights of Bragg grown in the presence and absence of nitrate and nts382 grown in the absence of nitrate were also significantly increased by the application of CCC. In contrast, nts382 plus nitrate and CCC showed no increase, perhaps suggesting that the optimal growth under these conditions has been reached.

The initial steps in the biosynthesis of gibberellins and also of the naturally occuring growth inhibitor abscisic acid involve a common pathway from mevalonic acid (see figure 4.2). Thus the involvement of ABA in nodulation control must not be discarded, since a blockage on one side of these connected pathways would naturally affect the other. The synthetic growth retardant CCC [(2-chloroethyl)trimethyl-ammonium-chloride] specifically inhibits the cyclization of geranyl-geranyl pyrophosphate to (-) kaurene. As

geranyl-geranyl pyrophosphate is the final common precursor for both the ABA and gibberellin pathways, the application of CCC should specifically inhibit gibberellin synthesis. However, such a blockage could increase the substrate availability for the branched pathway leading to ABA. Hence CCC may not only lower the amounts of gibberellic acids but also increase ABA levels. This illustrates the complexity of inhibitor studies especially when the affected pathways are interactive.

4.3 EXPERIMENT 7 : EFFECT OF INTER-COTYLEDONARY INJECTIONS OF (A) GA_3 AND (B) IAA ON NODULATION AND PLANT GROWTH

4.3.1 INTRODUCTION

The previous experiment involved the use of foliar applications of growth regulators, and provided preliminary evidence for the involvement of gibberellic acid in nodule suppression. The following experiment involved the use of inter-cotyledonary injections of hormones. These are considered more reliable than foliar applications because a known amount of phyto-hormone can be injected into the cotyledon. Application of growth hormones via foliar sprays, provides a rapid method of investigating the effect of a particular substance, but present a problem in that the leaf cuticle shows some resistance to assimilation, and so the actual amount of hormone inside the plant may be variable. Additionally it is not known whether hormones are taken up equally by nts382 and Bragg after being treated with foliar sprays.

4.3.2 DESIGN

Seeds of Bragg and nts382 were planted in sterile pots (20 cm high x 20 cm top diameter) of sand/vermiculite (3:1) at a density of 15 per pot. Fourteen days after planting, the pots were thinned to 10 plants and inoculated with *B. japonicum* strain USDA110 (as per experiment 4.1). Inter-cotyledonary injections were begun immediately after inoculation.

The treatments used in this experiment involved injecting one of three concentrations of IAA or GA_3 into Bragg and nts382 plants watered with Herridge nutrient solution supplemented with either 0.5 mM KNO_3 (low nitrate) or 5.5 mM KNO_3 (high nitrate). Low nitrate was used as it had no apparent effect on nodulation and nitrogen fixation, but helped with the general fitness of the plants.

Each plant was injected 11 times between days 14 and 31 after seed sowing with one of the following amounts of hormone:

(i) 1 µg GA_3 per 20 µl injection (3.16×10^{-4} M)
(ii) 5 µg GA_3 per 20 µl injection (1.56×10^{-3} M)
(iii) 10 µg GA_3 per 20 µl injection (3.16×10^{-3} M)
(iv) 1 µg IAA per 20 µl injection (1.44×10^{-4} M)
(v) 5 µg IAA per 20 µl injection (7.21×10^{-4} M)
(vi) 10 µg IAA per 20 µl injection (1.44×10^{-4} M)
(vii) Control - no injection

N.B. A separate experiment in which water and ethanol (80 % : 20 %) was injected into cotyledons demonstrated no significant difference between plants which were injected with water : ethanol and those which were not injected (i.e. as in the control for this experiment) (see Experiment 8).

Plants were harvested on day 37, at which time nodules were detached and counted. Fresh weight measurements were made of roots, shoots, nodules, and stem length was also measured. Data was also expressed as specific nodule weight, nodule number per gram of fresh root, shoot, or total weight.

4.3.3 RESULTS: EXPERIMENT 7(A) : INTER-COTYLEDONARY INJECTIONS OF GA_3

Bragg + 0.5 mM KNO_3 + GA_3

Table 4.3 shows that 1, 5 and 10 µg GA_3 significantly reduced nodule number per plant by 38, 30, and 40 % respectively from the Bragg plus 0.5 mM KNO_3 control. All levels of GA_3 significantly reduced total nodule fresh weight per plant (table 4.4), which was partially compensated for by increased specific nodule fresh weights (table 4.7).

GA_3 had no effect on root weight (table 4.5) although it significantly increased stem length (table 4.11), there was no effect on shoot weight (table 4.6). Therefore it is proposed that GA_3 has a direct effect on the suppression of nodule number in wild-type soybean grown on low level nitrate.

Bragg + 5.5 mM KNO_3 + GA_3

Low levels of GA_3 (1µg) significantly increased nodule number, but higher levels (5 and 10 µg) reduced nodule number per plant (table 4.3). There was little effect on nodule fresh weight per plant in general (table 4.4), but a slight increase in specific nodule weight was noted with the 10 µg treatment (table 4.7). However, all levels of GA_3 significantly reduced root fresh weight (table 4.5), and increased stem elongation (Table 4.11). Shoot fresh weight was increased significantly by the 1 µg treatment (table 4.6).

Nts382 + 0.5 mM KNO_3 + GA_3

All levels of GA_3 application significantly reduced nodule number per plant (table 4.3), but had little effect on total nodule fresh weight per plant (table 4.4). The plant compensated for this reduced nodule number by significantly increasing the specific nodule fresh weight (table 4.7) with all levels of applied GA_3. The treatments described here had no effect on root fresh weight (table 4.5), but all levels significantly increased

Table 4.3: Effect of inter-cotyledonary injections of IAA and GA_3 on nodule number / plant (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL (No injection)	63 ± 9^a	23 ± 3^a	408 ± 41^a	521 ± 92^a
1 µg IAA	24 ± 5^b	31 ± 17^a	361 ± 37^a	384 ± 73^b
5µg IAA	19 ± 8^b	16 ± 10^a	197 ± 15^b	249 ± 60^b
10 µg IAA	25 ± 8^b	27 ± 15^a	276 ± 61^b	176 ± 53^b
LSD 0.05	11	18	54	55
1 µg GA_3	24 ± 7^b	35 ± 7^b	141 ± 46^b	393 ± 126^b
5 µg GA_3	14 ± 10^b	12 ± 3^b	181 ± 45^b	258 ± 88^b
10 µg GA_3	15 ± 5^b	8 ± 2^b	146 ± 47^b	175 ± 71^b
LSD 0.05	12	6	56	114

Nodule number per plant is expressed as $\bar{x} \pm$ s.d.
Numbers in the same column with different alphabetic superscripts are significantly different from the control, as determined by the LSD 0.05. Treatments are as described in section 4.3.2

Table 4.4 **Effect of inter-cotyledonary injections of IAA and GA_3 on total nodule fresh weight / plant (Experiment 7)**

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	0.20 ± 0.07^{a}	0.06 ± 0.04^{a}	0.65 ± 0.05^{a}	1.12 ± 0.21^{a}
1µg IAA	0.16 ± 0.06^{a}	0.04 ± 0.03^{a}	0.76 ± 0.10^{a}	0.92 ± 0.21^{a}
5 µg IAA	0.09 ± 0.04^{b}	0.07 ± 0.04^{a}	0.70 ± 0.14^{a}	0.64 ± 0.09^{b}
10 µg IAA	0.18 ± 0.07^{a}	0.06 ± 0.02^{a}	0.74 ± 0.14^{a}	0.62 ± 0.15^{b}
LSD 0.05	0.07	0.045	0.13	0.21
1 µg GA_3	0.14 ± 0.07^{a}	0.09 ± 0.04^{b}	0.35 ± 0.08^{b}	0.71 ± 0.24^{b}
5 µ GA_3	0.08 ± 0.05^{b}	0.04 ± 0.01^{a}	0.52 ± 0.14^{a}	0.37 ± 0.11^{b}
10 µg GA_3	0.07 ± 0.01^{b}	0.05 ± 0.02^{a}	0.64 ± 0.12^{a}	0.29 ± 0.15^{b}
LSD 0.05	0.06	0.03	0.14	0.21

Total nodule weight (g) per plant expressed as $\bar{x} \pm$ s.d; Numbers in the same column with different alphabetic superscripts are significantly different from the control as determined by the LSD 0.05. Treatment levels are as described in section 4.3.2.

Table 4.5 Effect of inter-cotyledonary injections of IAA and GA_3 on root fresh weight * (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	2.06 ± 0.49^a	4.18 ± 0.99^a	1.85 ± 0.21^a	1.62 ± 0.54^a
1 µg IAA	2.22 ± 1.14^a	2.54 ± 1.08^b	2.13 ± 0.38^a	1.68 ± 0.46^a
5 µg IAA	1.69 ± 0.39^a	2.77 ± 0.62^b	0.88 ± 0.16^b	1.48 ± 0.16^a
10 µg IAA	2.19 ± 0.60^a	2.75 ± 0.65^b	1.63 ± 0.39^a	1.77 ± 0.44^a
LSD 0.05	0.87	1.03	0.41	0.54
1 µg GA_3	2.08 ± 0.70^a	3.02 ± 0.69^b	1.56 ± 0.34^a	1.13 ± 0.15^a
5 µg GA_3	1.60 ± 0.49^a	2.99 ± 0.93^b	1.43 ± 0.35^a	1.47 ± 0.37^a
10 µg GA_3	2.07 ± 0.25^a	1.71 ± 0.17^b	2.14 ± 0.36^a	1.95 ± 0.65^a
LSD 0.05	0.65	0.90	0.41	0.58

Root fresh weight (g) expressed as $\bar{x} \pm$ s.d.; Numbers in the same columns with different alphabetic superscripts are significantly different as determined by the LSD 0.05., Treatments are as described in section 4.3.2.
Root fresh weight does not include nodule fresh weight.

Table 4.6 Effect of inter-cotyledonary injections of IAA and GA_3 on shoot fresh weight (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	4.15 ± 0.59^a	5.74 ± 0.91^a	4.34 ± 0.94^a	4.55 ± 0.99^a
1 µg IAA	4.73 ± 1.12^a	5.29 ± 1.38^a	3.43 ± 0.93^a	3.81 ± 0.70^a
5 µg IAA	3.26 ± 0.79^a	4.18 ± 0.88^b	4.73 ± 0.72^a	3.07 ± 0.69^b
10 µg IAA	5.11 ± 1.28^a	4.50 ± 0.83^a	4.00 ± 0.49^a	3.49 ± 0.80^b
LSD 0.05	1.21	1.28	0.99	1.01
1 µg GA_3	4.47 ± 1.29^a	6.94 ± 0.73^b	2.20 ± 0.54^b	3.93 ± 0.45^a
5 µg GA_3	3.39 ± 1.43^a	6.53 ± 1.02^a	3.57 ± 0.66^a	3.91 ± 0.64^a
10 µg GA_3	5.07 ± 0.72^a	5.65 ± 1.45^a	3.53 ± 0.82^a	4.37 ± 1.27^a
LSD 0.05	1.33	1.40	0.95	1.07

Shoot fresh weight (g) expressed as $\bar{x} \pm$ s.d., Numbers in the same column with different alphabetic superscripts are significantly different as determined by the LSD 0.05., Treatments are as described in section 4.3.2.

Table 4.7 Effect of inter-cotyledonary injections of IAA and GA_3 on specific nodule weight (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	3.51 ± 1.74^{a}	3.06 ± 1.09^{a}	1.62 ± 0.42^{a}	2.09 ± 0.26^{a}
1 µg IAA	5.93 ± 2.28^{b}	2.16 ± 0.70^{a}	2.11 ± 0.40^{a}	2.23 ± 0.44^{a}
5 µg IAA	5.13 ± 0.91^{a}	2.53 ± 0.27^{a}	3.65 ± 0.64^{b}	2.64 ± 0.90^{b}
10 µg IAA	6.91 ± 1.29^{b}	2.39 ± 0.80^{a}	2.70 ± 0.51^{b}	3.17 ± 0.50^{b}
LSD 0.05	2.30	0.92	0.54	0.54
1 µg GA_3	5.71 ± 1.57^{b}	2.49 ± 0.79^{a}	2.77 ± 0.60^{b}	1.81 ± 0.17^{a}
5 µg GA_3	5.65 ± 1.47^{b}	3.10 ± 0.80^{a}	2.93 ± 0.70^{b}	1.36 ± 0.19^{b}
10 µg GA_3	5.88 ± 1.92^{b}	4.30 ± 1.70^{b}	4.92 ± 0.11^{b}	1.52 ± 0.36^{b}
LSD 0.05	2.13	1.58	0.90	0.32

Specific nodule fresh weight (mg) expressed as $\bar{x} \pm$ s.d. Numbers in the same column with different alphabetic superscripts are significantly different as determined by the LSD 0.05., Treatments are as described in section 4.3.2.

Table 4.8 Effect of inter-cotyledonary injections of IAA and GA_3 on nodule number / g root weight (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	28 ± 14^a	6 ± 2^a	222 ± 15^a	296 ± 55^a
1 µg IAA	13 ± 4^b	12 ± 5^b	165 ± 24^b	247 ± 101^a
5 µg IAA	12 ± 6^b	8 ± 3^a	224 ± 30^a	246 ± 92^a
10 µg IAA	9 ± 3^b	9 ± 4^b	173 ± 46^b	94 ± 29^b
LSD 0.05	8	5	38	81
1 µg GA_3	13 ± 6^b	11 ± 4^b	105 ± 6^b	337 ± 133^a
5 µg GA_3	10 ± 6^b	4 ± 2^a	133 ± 42^b	172 ± 86^b
10 µg GA_3	8 ± 3^b	4 ± 1^a	69 ± 21^b	88 ± 34^b
LSD 0.05	9	3	34	99

Data expressed as $\bar{x} \pm$ s.d. Weights expressed in mg. fresh weight.
Numbers in the same column with different alphabetic superscripts are significantly different as determined by the LSD 0.05; Treatments are as described in section 4.3.2.

Table 4.9 Effect of inter-cotyledonary injections of IAA and GA_3 on nodule number / g shoot weight (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	11.07 ± 4.65^a	3.92 ± 1.22^a	86 ± 20^a	119 ± 35^a
1 µg IAA	5.31 ± 1.11^b	6.03 ± 2.77^b	110 ± 48^a	102 ± 21^a
5 µg IAA	5.34 ± 1.65^b	4.79 ± 2.07^a	57 ± 23^a	78 ± 23^b
10 µg IAA	4.47 ± 0.79^b	6.15 ± 1.99^b	71 ± 27^a	23 ± 16^b
LSD 0.05	3.3	2.7	43	31
1 µg GA_3	5.64 ± 2.05^b	6.17 ± 2.24^b	71 ± 27^a	102 ± 25^a
5 µg GA_3	4.14 ± 2.12^b	1.35 ± 0.44^b	49 ± 16^a	69 ± 19^b
10 µg GA_3	3.06 ± 0.96^b	1.59 ± 0.55^b	41 ± 17^a	43 ± 16^b
LSD 0.05	3.5	1.6	26	31

Data expressed as $\bar{x} \pm$ s.d.
Numbers in the same column with differenct alphabetic superscripts are significantly different as determined by the LSD 0.05; Treatments are as described in section 4.3.2.

Table 4.10 Effect of inter-cotyledonary injections of IAA and GA_3 on nodule number / g total plant weight (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	7.61 ± 0.13^a	2.47 ± 0.76^a	54 ± 10^a	63 ± 18^a
1 µg IAA	3.29 ± 0.86^b	2.96 ± 1.36^a	45 ± 32^a	60 ± 22^a
5 µg IAA	3.65 ± 1.58^b	3.30 ± 1.10^a	26 ± 5^b	44 ± 13^b
10 µg IAA	3.38 ± 1.05^b	3.64 ± 1.75^a	36 ± 14^b	27 ± 8^b
LSD 0.05	1.60	1.67	18	19
1 µg GA_3	3.58 ± 1.00^b	3.39 ± 0.89^a	32 ± 12^b	56 ± 14^a
5 µg GA_3	4.29 ± 1.97^b	1.24 ± 0.46^b	26 ± 11^b	37 ± 12^b
10 µg GA_3	1.96 ± 0.60^b	1.11 ± 0.34^b	27 ± 14^b	24 ± 9^b
LSD 0.05	1.56	0.80	15	18

Data expressed as $\bar{x} \pm$ s.d.
Numbers in the same column with different alphabetic superscripts are significantly different as determined by the LSD 0.05; Treatments are as described in section 4.3.2.

Table 4.11 Effect of inter-cotyledonary injections of IAA and GA_3 on stem length (Experiment 7)

Treatment	Bragg + 0.5 mM KNO_3	Bragg + 5.5 mM KNO_3	nts382 + 0.5 mM KNO_3	nts382 + 5.5 mM KNO_3
CONTROL	31 ± 4^a	36 ± 2^a	34 ± 2^a	28 ± 4^a
1 µg IAA	38 ± 2^b	36 ± 2^a	34 ± 3^a	35 ± 3^b
5 µg IAA	33 ± 5^a	36 ± 2^a	40 ± 3^a	33 ± 3^b
10 µg IAA	40 ± 2^b	37 ± 5^a	36 ± 1^a	37 ± 2^b
LSD 0.05	5	8	4	3
1 µg GA_3	44 ± 3^b	54 ± 9^b	42 ± 1^b	42 ± 2^b
5 µg GA_3	55 ± 9^b	60 ± 9^b	67 ± 12^b	49 ± 9^b
10 µg GA_3	80 ± 7^b	67 ± 13^b	64 ± 7^b	63 ± 4^b
LSD 0.05	9	12	7	6

Data expressed as $\bar{x} \pm$ s.d; Stem length measured in cm.
Numbers in the same column with different alphabetic superscripts are significantly different as determined by the LSD 0.05; Treatments are as described in section 4.3.2.

stem length, while reducing shoot fresh weight, (table 4.6), although only the 1 µg result was significant.

Nts382 + 5.5 mM KNO_3 + GA_3

All levels of GA_3 significantly reduced the nodule number per plant (table 4.3) and nodule weight per plant (Table 4.4). Specific nodule weight was less than the control (table 4.7) with suppression by the 5 and 10 µg treatment being significant. Root (table 4.5) and shoot (table 4.6) fresh weights were not affected by GA_3 (however stem length was increased significantly as shown in table 4.11). The results indicate a direct effect of this hormone on nodulation.

4.3.4 DISCUSSION : EFFECT OF GA_3 INJECTIONS ON NODULATION AND PLANT GROWTH

Data from experiment 8 (section 4.4) show that the controls used in these experiments (i.e. non-injected plants) are valid since there was no significant difference between nodule number, root, shoot or nodule fresh weight of non-injected plants compared to those treated with sham injections of ethanol : water.

Injections of GA_3 significantly reduced nodule number of both Bragg and nts382 plants grown in the presence and absence of nitrate, however nodule development was affected in different ways. Bragg plants grown on low level nitrate demonstrated a significantly reduced nodule mass, which was only partially compensated for by an increase in specific nodule weight. In the presence of higher levels of nitrate, nodule number suppression was compensated for more fully by increases in specific nodule weight. Similarly, nts382 plants grown in the presence of 0.5 mM KNO_3, and treated with GA_3, demonstrated nodule weight compensation through the production of larger nodules, however, the reduction in nodule number of nts382 nodules grown in the presence of 5.5 mM KNO_3, was not compensated for, with smaller nodules developing.

Suppression of root fresh weight was observed only on Bragg plants grown in the presence of 5.5 mM KNO_3 after GA_3 injection, with the other plants showing no change. GA_3 injections had no effect on the shoot weight of any of the treatments, but stem length was significantly increased on all plants.

4.3.5 RESULTS: EXPERIMENT 7(B): EFFECT OF INTER-COTYLEDONARY INJECTIONS OF IAA

Bragg + 0.5 mM KNO_3 + IAA

Table 4.3 shows that Bragg plants grown under low nitrate conditions (0.5 mM KNO_3) developed 63 ± 9 nodules per plant. The injection of 1 µg IAA to a similar set of plants resulted in a significant suppression of nodulation to 24 ± 5 nodules (or 26 % of the control). Tenfold higher concentrations of IAA had no further influence on nodule suppression.

None of the concentrations of IAA had a significant effect on root (table 4.5) or shoot (table 4.6) fresh weight of low nitrate treated plants. Similarly, the injection of 1 or 10 µg IAA had no significant effect on nodule fresh weight per plant (table 4.4), although 5 µg significantly reduced it. Specific nodule fresh weight (table 4.7) and stem length (table 4.11) were increased significantly by 1 and 10 µg injections. The 5 µg IAA injection caused increases in specific nodule fresh weight and stem length however these differences were not statistically significant.

The expression of nodule number per gram of fresh root (table 4.8) or shoot (table 4.9) or total weight (table 4.10) showed a significant reduction upon the application of 1, 5 or 10 µg IAA.

Bragg + 5.5 mM KNO_3 + IAA

Bragg control plants, watered with a nutrient solution containing 5.5 mM KNO_3, developed 23 ± 3 nodules, illustrating nitrate inhibition of nodule number. When 1-10 µg IAA was injected into similar sets of plants there was no significant difference in the mean nodule number per plant (table 4.3), total nodule weight per plant (table 4.4), specific nodule weight (table 4.7) or stem length (table 4.11). All levels of IAA significantly suppressed the fresh root weight (Table 4.5), and all tested levels of IAA demonstrated significant suppression of shoot fresh weight.

Nts382 + 0.5 mM KNO_3 + IAA

When nts382 control plants were grown under low nitrate conditions, they developed a mean nodule number per plant of 408 ± 41. Treatment with 1 µg IAA had no effect on nodulation, but higher levels of IAA significantly reduced nodule number per plant (table 4.3) without affecting the total nodule fresh weight per plant (table 4.4) That is, the reduction in nodule number was compensated for by an increase in specific nodule fresh weight (table 4.7).

Table 4.5 shows that 1 and 10 µg IAA had no effect on root fresh weight and table 4.6 shows that 5 and 10 µg IAA had no effect on shoot fresh weight. None of the levels of IAA tested had an effect on stem length (Table 4.11), except the 5 µg treatment which cannot be explained.

Nts382 + 5.5 mM KNO_3 + IAA

Nts382 plants grown in the presence of 5.5 mM KNO_3, developed 571 ± 92 nodules per plant, or 27 % more than low nitrate treated plants, thus showing a degree of nitrate tolerance. When similar plants were treated with IAA there was a correlation between the concentration of hormone injected and the degree of nodule suppression observed. 1, 5 and 10 µg IAA suppressed nodulation to 74 %, 48 % and 34 % of the control respectively (table 4.3). The total nodule weight per plant was suppressed significantly by the addition of 5 or 10 µg IAA (table 4.4), and specific nodule weight was significantly reduced by the addition of 5 and 10 µg IAA (table 4.7). These results demonstrate that IAA causes suppression of both nodule initiation and nodule development.

The application of 1 - 10 µg IAA had no significant effect on root weights (table 4.5), but 5 - 10 µg IAA significantly reduced shoot fresh weights (table 4.6). Stem length was also significantly increased by the application of 1-10 µg IAA (table 4.11).

4.3.6 DISCUSSION: EFFECT OF IAA INJECTIONS ON NODULATION AND PLANT GROWTH

When IAA was applied to Bragg plants by inter-cotyledonary injection, the concentration of nitrate in the nutrient solution (either low: 0.5 mM KNO_3 or high: 5.5 mM KNO_3) was observed to alter the nodulation response.

The application of IAA to Bragg plants grown in the presence of low nitrate concentration resulted in a significant suppression of the total nodule number per plant but total nodule weight remained constant regardless of applications of 1 and 10 µg IAA (5 µg IAA proved an exception, possibly due to experimental error), consequently specific nodule weight increased significantly. Indeed, the application of 10 µg IAA increased specific nodule fresh weight by 97 %. Thus IAA treatment of low nitrate grown Bragg plants resulted in fewer but larger nodules. This provides evidence for the "compensation" argument proposed by Nutman (1952) in which he suggested that the plant regulates the amount of nodule tissue whether it be by initiating more nodules or by allowing the continued growth and extension of existing nodules.

The degree of nodule suppression by IAA observed on low nitrate grown Bragg plants was equal to that by high nitrate alone. Further, the combination of IAA and 5.5 mM KNO_3 produced no further suppression of nodule number, and had no effect on the nodule number, total nodule weight or specific nodule weight This indicates that Bragg plants grown on high nitrate are insensitive to IAA.

However, these data do not support the results from Valera and Alexander (1965) who showed that IAA (10^{-8} M) could reverse the nitrate inhibition of nodulation in lucerne.

IAA treatment of low nitrate grown plants had no effect on root or shoot fresh weight, indicating that IAA has a direct effect on nodulation rather than indirectly through alterations of plant growth. Conversely, the addition of 1 - 10 µg IAA significantly reduced the root weight of Bragg plants grown in high nitrate and 5 - 10 µg significantly reduced the shoot weight without suppressing nodulation.

This means that, in the presence of high nitrate, IAA may cause a reduction in several plant growth parameters which are not demonstrated in low nitrate grown plants. To take into account these variations, nodule number was expressed as function of plant fresh weight. One thus has two choices for data analysis; express the nodulation parameter per plant (and overlook the secondary plant effects) or express the nodulation data as a function of plant fresh weight, which includes the secondary effects but thereby assumes a linear correlation between say, root development and nodule number). This latter assumption is laden with traps as is the first alternative, but perhaps by recognizing this and dealing with both, we can generate some testable hypotheses.

Nts382 plants grown under low nitrate conditions and treated with IAA, like similarly grown Bragg plants, demonstrated nodule compensation, with nodule number decreasing, nodule weight staying constant and specific nodule fresh weight increasing. IAA showed no effects on root or shoot fresh weight or stem length so it is proposed that IAA had a direct effect on suppressing the number of initiated nodules. The increase in specific nodule fresh weight may therefore be a compensation effect similar to the one discussed above (cf. Nutman 1952).

When nts382 plants were grown under high nitrate conditions, IAA significantly reduced the nodule number and total nodule weight , and while there was an increase in specific nodule weight, it was not high enough to compensate for the reduction in total nodule weight. Thus in the presence of IAA, it is proposed that nitrate interfers with plant metabolism such that the nodule compensation mechanism is suppressed.

4.3.7 DISCUSSION: COMPARISON OF THE EFFECT OF NITRATE ON NTS382 AND BRAGG PLANT GROWTH AND NODULATION

Table 4.12 examines that changes in nodulation and plant growth parameters of Bragg and nts382 plants grown in the presence of 5.5 mM KNO_3 as compared to their 0.5 mM KNO_3 treated controls. The table has been generated from the control data in tables 4.3, 4.4, 4.5, 4.6, 4.7 and 4.11. It is of interest to note that for Bragg and nts382, the responses of each parameter show opposite trends.

In the presence of high level nitrate, nodule number of Bragg is reduced 55 % while that of nts382 is increased 28 %, thus demonstrating that Bragg is inhibited by nitrate, while nts382 is nitrate tolerant. Additionally, nitrate suppresses the development of nodules in Bragg, but causes an increase in specific nodule weight on nts382 plants.

Bragg roots weights were increased to 104 % of their low nitrate treated control plants, while nts382 plants demonstrated a 12 % reduction. Bragg shoot weight, was increased by 40 % whereas nts382 shoot weight was only increased 5 %.

Leaf area and stem length were both increased (30 % and 17 % respectively),when Bragg plants were grown in the presence of high level nitrate, but nts382 plants resulted in reductions of 18 % and 17 % respectively.

These data suggest that nitrate, assimilated via the roots may be transfered to other plant parts and there influence specific physiological processes, resulting in changes in shoot growth, leaf area, stem length. Additionally, it may act locally to affect nodule initiation, development and root growth. As yet, it is not understood why nts382 and Bragg respond differently to assimilated nitrate. It is possible that nitrate may act as a morphogen, since it has been shown to act as both a stimulator as well as an inhibitor of plant growth. Additionally, nitrate may interfer with the sensitivity of hormone receptors to specific phyto-hormones, or may alter the concentration of hormone binding sites. These ideas will be further discussed in chapter 6.

TABLE 4.12 Effect of nitrate (5.5 mM KNO3) on Bragg and nts382 nodulation and plant growth parameters expressed as a percentage of 0.5 mM KNO3 control plant data (From tables 4.3 to 4.11)

PARAMETER	BRAGG + 5.5 mM KNO_3	nts382 + 5.5 mM KNO_3
Nodule No.	Reduced 55 %	Increased 28 %
Nodule wt. (g)	Reduced 63 %	Increased 64 %
Specific Nod. wt.	Reduced 17 %	Increased 28 %
Root weight (g)	Increased 104 %	Reduced 12 %
Shoot weight (g)	Increased 40 %	Increased 5 %
Leaf Area (cm^2)	Increased 30 %	Reduced 18 %
Stem length (cm)	Increased 17 %	Reduced 17 %

Table 4.13 : Effect of nitrate (5.5 mM KNO_3) on Bragg and nts382 nodulation and plant growth parameters as compared to 0.5 mM KNO_3 control plant data.

4.4 EXPERIMENT 8: EFFECT OF CONTROL INJECTIONS OF ETHANOL AND WATER ON SOYBEAN NODULATION AND PLANT GROWTH

4.4.1 INTRODUCTION

The following experiment was designed to investigate whether the mechanical injury caused to cotyledons after injection of phyto-hormones, had a role in the observed nodule suppression. Bragg plants were either grown without injections, or their cotyledons were injected with a mixture of water and ethanol. This was the same carrier mixture used in phyto-hormone injections.

4.4.2 DESIGN

Nts382 and Bragg soybeans were grown separately in sterile pots of sand: vermiculite for 10 days prior to inoculation with *B.japonicum* strain USDA110. Half of the plants were injected in one of their cotyledons with 10 µl of an ethanol / d.H_20 mixture (20 % : 80 %) and the other half were left un-injected. Eleven injections were made between days 14 and 31 after planting. Plants were harvested on day 33 and nodule number, nodule, root and shoot fresh weights were recorded.

4.4.3 RESULTS

Table 4.13 shows nodule number, root, shoot and nodule fresh weights for plants that were injected with control treatments of ethanol and water, compared to plants that were not injected. This experiment showed that there was no statistical difference in the nodulation or growth parameters of Bragg plants which had been mechanically injured through inter-cotyledonary injections. Further the injection of ethanol was not damaging to the plant's well being. Thus, it was considered acceptable to use non injected plants as controls for experiments in which hormones were injected into the cotyledons of soybean plants.

Table 4.13 Effect of inter-cotyledonary injections of ethanol / d.$H_2$0 on nodulation and growth of Bragg plants (Experiment 8)

Treatment	Nodule Number	Root fresh wt. (g)	Shoot fresh wt. (g)	Nodule fresh wt. (g)
Injected (20 % Ethanol 80 % water)	44 ± 8	4.91 ± 0.65	5.18 ± 0.12	0.74 ± 0.20
Non-injected	44 ± 18	5.86 ± 0.84	4.56 ± 0.24	0.55 ± 0.11
LSD 0.05	24	1.23	0.63	0.26

Data expressed as $\bar{x} \pm$ s.d; Treatments are as described in section 4.4.2.

4.5 EXPERIMENT 9 : INTER-COTYLEDONARY INJECTION OF GA_4 ON NODULATION AND PLANT GROWTH

4.5.1 INTRODUCTION

The involvement of gibberellic acid in the suppression of nodulation in soybean was further investigated through GA_4 injections. The pathway of gibberellin biosynthesis is presented in figure 4.2. GA_4 was chosen as it has been shown to be the the immediate precursor of GA_1 and GA_7 and it had previously been shown by Williams and de Mallorca (1984), that foliar applications (3.01×10^{-5} M) delayed *Glycine soya* nodulation and reduced numbers, size and effectiveness, without apparent effects on root or shoot growth.

Thus it was considered of interest to investigate the effect of this phyto-hormone on the nodulation of both the wild-type cultivar Bragg and its nts382 autoregulation mutant.

4.5.2 DESIGN

Glycine max cv. Bragg and nts382 were grown in pots of sterilized vermiculite (density of 10 plants) for 7 days prior to inoculation with *B. japonicum* strain USDA110 (10^9 viable cells / pot, using peat inoculum). Plants were watered daily with Herridge nutrient solution supplemented with low level nitrate (0.5 mM KNO_3). Between days 7 and 24 each plant received a daily 20 µl injection of one of 5 concentrations of GA_4 as shown below:

(i) 1 ng GA_4 per 20 µl injection (1.44×10^{-7} M)
(ii) 10 ng GA_4 per 20 µl injection (1.44×10^{-6} M)
(iii) 0.1 µg GA_4 per 20 µl injection (1.44×10^{-5} M)
(iv) 1 µg GA_4 per 20 µl injection (1.44×10^{-4} M)
(v) 5 µg GA_4 per 20 µl injection (7.20×10^{-4} M)
(vi) Control - no injection

Plants were harvested at day 28 and nodules were detached and counted and root and shoot fresh weights were measured.

4.5.3 RESULTS: EXPERIMENT 9 - EFFECT OF INTER-COTYLEDONARY INJECTIONS OF GA_4 ON NODULATION AND PLANT GROWTH.

Table 4.14 shows data from Bragg and nts382 plants, grown in the presence of 0.5 mM KNO_3, injected with GA_4 (methyl-ester) in the range 1 ng to 5 µg daily. The most important observation is that nts382 plants were sensitive to GA_4 treatments while Bragg plants were not. Indeed, the treatments caused no significant effect on Bragg nodule number per plant, total nodule fresh weight per plant, root fresh weight or shoot fresh weight. When nts382 plants were grown under the same conditions, a significant reduction in nodule number per plant was observed. Injection of 10 ng GA_4 suppressed nodule number from 375 ± 43 to 300 ± 34 nodules without sigificantly reducing the total nodule weight per plant.

The plant compensated for this by generating a slight increase in specific nodule fresh weight from 2.1 ± 0.6 g to 2.6 ± 0.6 g (table 4.15), however the increase was not statistically significant. At higher levels of GA_4 (100 ng to 5 µg), nts382 nodule number was especially suppressed. At the same time nodule fresh weight was slightly (but not significantly) suppressed. One exception to this was the 1 µg injection, which did cause a significant decrease. This resulted in a slight compensatory, increase in specific nodule fresh weight.

Nts382 root fresh weight was not significantly affected by any of the GA_4 treatments except the 1 µg injection which resulted in an increase. Low concentrations of GA_4 (1 and 10 ng) had no significant effect on shoot fresh weight, however 100 ng, 1 µg and 5 µg injections significantly reduced it. Thus a correlation between GA_4 addition and nts382 nodule number suppression was observed, as shown previously for GA_3 injected plants.

Table 4.14 Effect of intercotyledonary injections of GA_4 on soybean nodulation and plant growth (Experiment 9)

Treatment	Nodule Number	Nodule fresh wt. (g)	Root fresh wt. (g)	Shoot fresh wt.(g)
Bragg control	42 ± 17^a	0.18 ± 0.04^a	1.84 ± 0.48^a	2.59 ± 0.28^a
10^{-9}g GA_4	66 ± 19^a	0.13 ± 0.04^a	$1.77 + 0.65^a$	2.39 ± 0.40^b
10^{-8}g GA_4	45 ± 17^a	0.20 ± 0.09^a	2.27 ± 0.86^a	2.77 ± 0.86^a
10^{-7}g GA_4	44 ± 21^a	0.14 ± 0.12^a	2.24 ± 0.74^a	2.48 ± 0.76^a
10^{-6}g GA_4	39 ± 20^a	0.23 ± 0.07^a	2.30 ± 0.53^a	2.77 ± 0.34^a
5×10^{-6}g GA_4	41 ± 27^a	0.16 ± 0.10^a	2.23 ± 0.60^a	2.75 ± 0.48^a
LSD 0.05	27	0.12	0.96	0.19
nts 382				
Control	375 ± 43^a	0.84 ± 0.36^a	0.92 ± 0.27^a	1.73 ± 0.42^a
10^{-9}g GA_4	305 ± 61^b	0.74 ± 0.18^a	0.80 ± 0.25^a	1.89 ± 0.58^a
10^{-8}g GA_4	300 ± 34^b	0.83 ± 0.24^a	0.69 ± 0.24^a	1.51 ± 0.33^a
10^{-7}g GA_4	148 ± 56^b	0.62 ± 0.28^a	0.57 ± 0.10^a	1.32 ± 0.28^b
10^{-6}g GA_4	159 ± 35^b	0.43 ± 0.23^b	0.44 ± 0.14^b	1.27 ± 0.23^b
5×10^{-6}g GA_4	192 ± 32^b	0.56 ± 0.27^a	1.03 ± 0.46^a	1.44 ± 0.39^b
LSD 0.05	63	0.37	0.38	0.28

All data are expressed as $\bar{x} \pm$ s.d.
Numbers in the same column with different alphabetic superscripts are significantly different from the control as determined by the LSD 0.05. Plants werre grown in 0.5 mM KNO_3
Treatments are as described in section 4.5.2.

Table 4.15 Effect of inter-cotyledonary injections of GA_4 on soybean nodulation and plant growth (Experiment 9)

Treatment	Specific Nodule wt (mg)	Nodule No. / g. root	Nodule No. / g. shoot
Bragg control	4.3 ± 0.7^a	25 ± 12^a	16 ± 5^a
10^{-9}g GA_4	2.8 ± 1.2^b	41 ± 16^b	$28 + 8^b$
10^{-8}g GA_4	5.5 ± 3.3^b	25 ± 15^a	15 ± 6^a
10^{-7}g GA_4	3.4 ± 2.5^a	22 ± 10^a	17 ± 6^a
10^{-6}g GA_4	3.8 ± 2.6^a	18 ± 10^b	19 ± 3^a
5×10^{-6}g GA_4	4.0 ± 2.1^a	19 ± 12^b	17 ± 9^a
LSD 0.05	0.5	5	10
nts 382			
Control	2.1 ± 0.6^a	412 ± 140^a	220 ± 23^a
10^{-9}g GA_4	2.4 ± 0.7^a	404 ± 64^a	168 ± 51^a
10^{-8}g GA_4	2.6 ± 0.6	378 ± 103	202 ± 28^a
10^{-7}g GA_4	3.9 ± 0.9^b	258 ± 78^b	115 ± 45^b
10^{-6}g GA_4	2.6 ± 0.7^a	378 ± 118^a	142 ± 39^b
5×10^{-6}g GA_4	2.5 ± 0.8^b	286 ± 90^b	149 ± 37^a
LSD 0.05	1.1	52	54

Data are expressed as $\bar{x} \pm$ s.d.
Numbers in the same column with different alphabetic superscripts are significantly different from the control as determined by the LSD 0.05. Plants were grown in 0.5 mM KNO_3
Treatments are as described in section 4.5.2.

4.6 EXPERIMENT 10: EFFECT OF ABA INJECTIONS ON NODULATION AND PLANT GROWTH

4.6.1 INTRODUCTION

In general, abscisic acid is considered a growth inhibitor. It appears to antagonize the effects of auxins and gibberellins, and possibly of cytokinins, in various tests, and as such it may compete with one of these hormones for a specific enzyme (or hormone binding) site. Alternatively, it might antagonize the action of a growth promoting substance by inhibiting its biosynthesis, or promote its inactivation in a plant. Indeed it has been shown that ABA inhibits the synthesis of GA_4 in the gibberellin pathway (see Durley *et al.* 1976).

As previously discussed in section 4.1.3.2, exogenous applications of ABA have been identified as strong inhibitors of nodulation in *Pisum sativum* (Phillips 1971); *Faba vulgaris* (Bano and Hillman 1986), it was decided to investigate the involvement of this hormone in the nodule regulation phenomenon.

4.6.2 DESIGN

The following experiment was done in conjunction with Dr A.J. Krotzky of the Botany department, A.N.U.

Bragg and nts382 seeds were surface sterilized with 70 % ethanol and planted in pots of sterile sand : vermiculite at a density of 10 per pot. Plants were watered daily with sterile Herridge nutrient solution (1/4 strength for the first 2 weeks and full strength thereafter) supplemented with 5.5 mM KNO_3. Plants were inoculated with *B. japonicum* strain USDA 110 on day 9. Between days 9 and 21 plants were injected with 10 µl of one of the following concentrations of ABA:

(i)	Control : Injection of water and methanol (20 % methanol : 80 % d.d.H_20)
(ii)	10 ng ABA
(iii)	50 ng ABA
(iv)	100 ng ABA.
(v)	500 ng ABA
(vi)	1µg ABA
(vii)	5 µg ABA
(viii)	10 µg ABA
(ix)	50 µg ABA
(x)	100 µg ABA

As ABA injections stimulated the senescence of cotyledons, one of the cotyledons was removed and injections were made at the place of their connection to the stem. After the last injection plants were left for a further 24 hours prior to harvest. (i.e. day 22)

4.6.3 RESULTS: EXPERIMENT 10 - EFFECT OF ABA INJECTIONS ON NODULATION AND PLANT GROWTH

When Bragg plants, grown in the presence of 5.5 mM KNO_3 were injected daily with 100 ng ABA there was a significant suppression in nodulation, from 49 ± 8 to 40 ± 13 nodules (see table 4.16). However, nts382 plants, (grown under the same conditions), showed a higher degree of tolerance to ABA since 5 µg and above was required to significantly reduce nodulation from 949 ± 186 to 465 ± 83. These results demonstrated that nts382 was less susceptible to ABA induced nodule suppression than Bragg by a factor of 50.

There was a hundred fold difference in the susceptibility of Bragg and nts382 in regards to ABA suppression of root weight. Indeed while 50 ng ABA significantly suppresses Bragg root fresh weight (from 2.84 ± 0.37 g to 2.21 ± 0.46 g.), nts382 roots showed no such suppression until 5 µg ABA was injected, (at which time root fresh weight was suppressed from 2.14 ± 0.30 g to 1.61 ± 0.25 g.).

The shoot fresh weights of both Bragg and nts382 plants remained unaffected by 0 - 10 µg ABA, however shoot fresh weights were suppressed by 50 - 100 µg injections, and as such the ABA induced suppression of nodulation is apparently not related to shoot development. This agrees with results of Bano and Hillman (1986) for *Pisum sativum.*

In addition, Durley *et al.*(1976), showed that ABA could inhibit the conversion of GA_4 to GA_1 (which may be converted to GA_3) and so the inhibition of nodule number by ABA reported above could be caused in this manner. Further, the fact that 100 ng ABA significantly suppressed Bragg nodule number while 5000 ng was required to significantly suppress nts382 nodule number suggests that the mutant has a lower endogenous level of gibberellic acids past GA_4 than Bragg and therefore more abscisic acid.

If the GA_4 to GA_1 step is suppressed by ABA in soybean, then this would lead to a buildup of GA_4 precursors. From section 4.4 we know that GA_4 had no effect on Bragg nodulation but did suppress nts382 nodule number at all tested levels. Thus, the reason that a higher level of ABA was required to suppress nts382 than for Bragg nodule number could be that nodulated Bragg has higher levels of endogenous gibberellins due to the autoregulation mechanism. Nts382, then, (being an autoregulation mutant) has a

Table 4.16: Effect of ABA injections on nodulation and plant growth of cv. Bragg and nts382 (Experiment 10)

Treatment	Nodule Number	Root fresh wt. (g)	Shoot fresh wt. (g)	Nodule No. / g. root fresh wt.
Bragg control	49 ± 8	2.84 ± 0.37	3.14 ± 0.18	17 ± 4
10 ng	54 ± 16	2.80 ± 0.26	3.15 ± 0.18	19 ± 5
50 ng	44 ± 8	2.21 ± 0.46	3.22± 0.30	20 ± 5
100 ng	40 ± 13	2.10 ± 0.43	3.11 ± 0.40	21 ± 11
500 ng	38 ± 13	2.06 ± 0.29	3.25 ± 0.69	18 ± 7
1 µg	6 ± 3	2.03 ± 0.32	3.18 ± 0.44	3 ± 1
5 µg	4 ± 4	2.02 ± 0.66	3.08 ± 0.34	2 ± 2
10 µg	1 ± 1	1.67 ± 0.48	2.78 ± 0.41	0.6 ± 0.7
50 µg	0 ± 0	1.27 ± 0.38	1.79 ± 0.39	0 ± 0
100 µg	0 ± 0	1.14 ± 0.33	1.12 ± 0.41	0 ± 0
LSD 0.05	6	0.54	1.0	5.5
nts382 control	949 ± 186	2.14 ± 0.29	2.94 ± 0.45	450 ± 106
10 ng	1100 ± 294	2.32 ± 0.6	2.47 ± 0.28	486 ± 116
50 ng	1100 ± 213	2.41 ± 0.39	2.42 ± 0.48	461 ± 90
100 ng	965 ± 198	2.28 ± 0.42	2.24 ± 0.27	424 ± 57
500 ng	1011 ± 183	2.28 ± 0.31	2.40 ± 0.30	448 ± 94
1 µg	834 ± 122	2.15 ± 0.21	2.30 ± 0.25	392 ± 84
5 µg	465 ± 83	1.61 ± 0.25	2.41 ± 0.41	295 ± 73
10 µg	195 ± 45	1.11 ± 0.21	2.21 ± 0.39	178 ± 43
50 µg	3 ± 4	0.64 ± 0.16	0.95 ± 0.24	5 ± 7
100 µg	0 ± 0	0.60 ± 0.14	0.91 ± 0.32	0.60 ± 0.14
LSD 0.05	229	0.47	1.2	97

Data expressed as $\bar{x} \pm$ s.d.; All plants watered with Herridge nutrient solution supplemented with 5.5 mM KNO_3. LSD 0.05 = least significant difference (5 % level)
Treatments are as described in section 4.6.2.

lowered GA pool and hence requires a higher concentration of ABA to block the GA pathway in order to suppress nodulation.

4.7 EXPERIMENT 11: ^{14}C MEVALONIC ACID INCORPORATION INTO BRAGG AND NTS382 SHOOT TISSUE

4.7.1 INTRODUCTION

In the preceeding experiments, GA_3, GA_4 and ABA were shown to play a role in the suppression of nodulation in the autoregulation mutant nts382. The common precursor for both gibberellic acid and abscisic acid biosynthesis is mevalonic acid (figure 4.2), and as such, ^{14}C labelled mevalonate was injected into soybean shoots to provide a preliminary study of the difference in the incorporation of hormone intermediates in the wild-type and nts382 mutants, as shown by changes in the HPLC elution profile. This study was expanded to investigate the differences between inoculated and un-inoculated plants.

Mevalonic acid is the ultimate precursor of both gibberellins and abscisic acid (see figure 4.2), and so was used to investigate differences in the incorporation profiles in the shoots of (1) an autoregulating wild-type soybean (Bragg + inoculation); (2) its non-nodulated control; (3) an autoregulation (super-nodulating) mutant (nts382 + inoculum); and (4) its non-nodulated control.

4.7.2 DESIGN

The following experiment was done in conjunction with Dr A.J. Krotzky of the Botany department (ANU).

Nts382 and Bragg seeds were planted in pots of sterile sand and vermiculite (pots were surface sterilized in 3% sodium hypochlorite) at a density of 15 plants per pot. At this stage, half the plants were inoculated with 10^9 cells / pot of *B.japonicum* strain USDA 110. Plants were watered daily with Herridge solution supplemented with 5.5 mM KNO_3.

On day 14,15 and 16, 5 µl ^{14}C mevalonic acid (specific activity 1.9 GBq/ mM or 51.4 mCi/mM, total radioactivity / injection = 100 nCi) was injected into the stem at the point immediately below the first trifoliolate. On day 17 the plants were harvest.

The extraction protocol is shown overleaf:

(i) The leaves were removed and ground up anaerobically with PVP (50 % of fresh leaf weight) and liquid nitrogen,
(ii) extracted in 80 % methanol (1:10 dry weight volume, i.e. 10 ml methanol to 1 g fresh weight leaves), overnight, under N_2,
(iii) the methanol was rotor vapored off,
(iv) the aqueous phase was pH adjusted to 2.5 - 3.0 and partitioned against N- hexane three times,
(v) pH adjusted to 9.0 and partitioned against ethyl acetate 3 times,
(vi) the ethyl acetate fraction was rotor vapored to dryness, then re-dissolved in 1 ml methanol (this now contained 80% of the radioactivity),
(vii) 20 - 50 µl aliquots were analysed on the Waters HPLC (reverse phase LL [low load] C18 column- ODS- 10 spheresorb). Run in an exponential gradient of ethyl acetate: methanol (30:70),
(viii) fractions were collected at 30 second intervals in a micro-centrix fraction collector,
(ix) an aliquot of each fraction was analysed on a Beckman liquid scintillation counter,
(x) The count data was keyed into a computer and printed out as cpm versus fraction number (see figure 4.3).

4.7.3 RESULTS: EXPERIMENT 11 - ^{14}C MEVALONIC ACID INCORPORATION INTO BRAGG AND NTS382 SOYBEAN SHOOTS

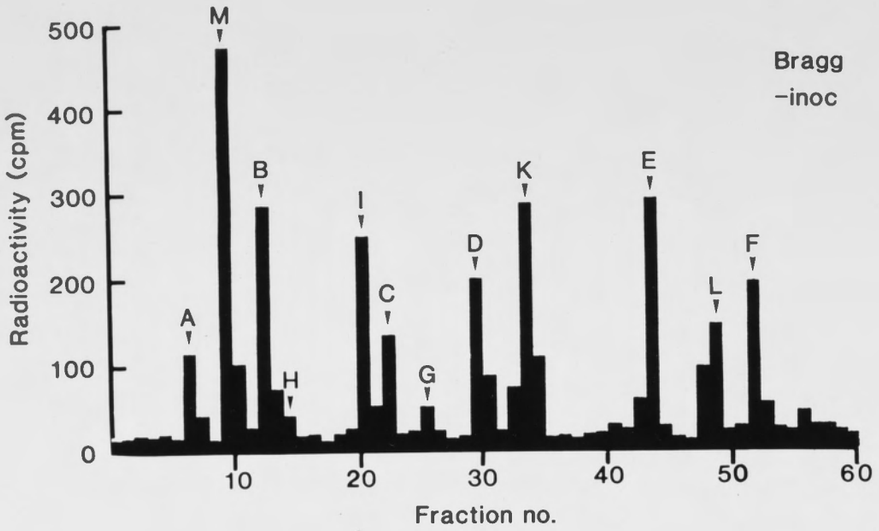
Figure 4.3 shows the histograms of the (cpm) per 30 second fraction for soybean shoots injected with ^{14}C - mevalonic acid. The four graphs show Bragg and nts382 grown in the presence and absence of *B. japonicum* strain USDA110.

The overall interpretation is that Bragg shows a simplified incorporation profile upon inoculation. In particular, inoculated Bragg plants are missing a peak at fraction 31(peak D), which is present in un-inoculated Bragg and nts382 as well as un-inoculated nts382 plants. In addition the peak at fraction 22 (peak C) on the inoculated Bragg profile appears to be less than on the three other systems. In terms of similarities, both Bragg and nts382 un-inoculated plants show a peak at fraction 7 (peak A) which is absent (or suppressed) upon inoculation.

As yet these peaks have not been identified. Indeed, the observed changes may only be associated with general nodule development, rather than being associated with the autoregulation phenomenon. Further analysis using HPLC techniques was considered beyond the scope of this thesis, but it is envisaged that this line of investigation will be continued by Dr A. Krotzky, in the near future.

Figure 4.3 **Incorporation profile of ^{14}C - mevalonic acid into Bragg and nts382 shoots (Experiment 11)**

The four histograms show data from inoculated and un-inoculated Bragg and nts382 plants grown in the presence of 5.5 mM KNO_3.


Bragg
−inoc
Radioactivity (cpm)
Fraction no.
0
100
200
300
400
500
10
20
30
40
50
60
A
B
C
D
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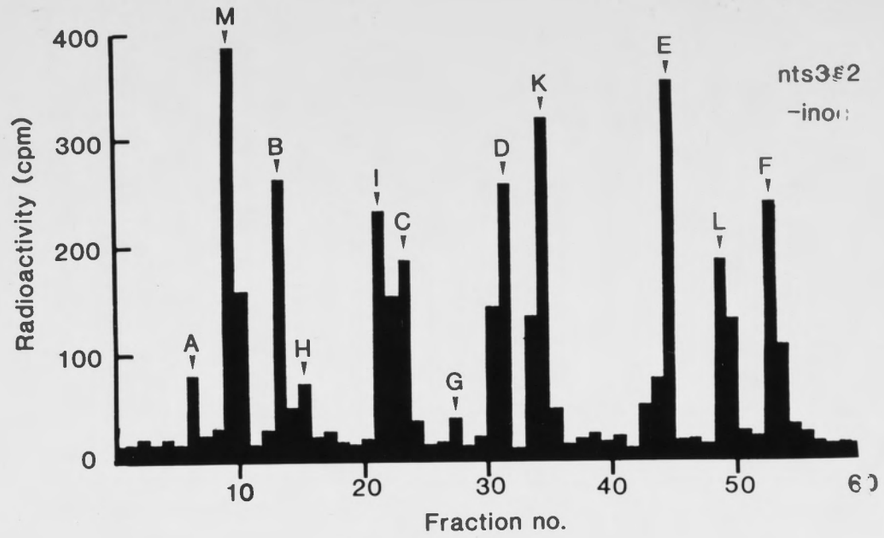
nts382
−inoc
Radioactivity (cpm)
Fraction no.
0
100
200
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400
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A
B
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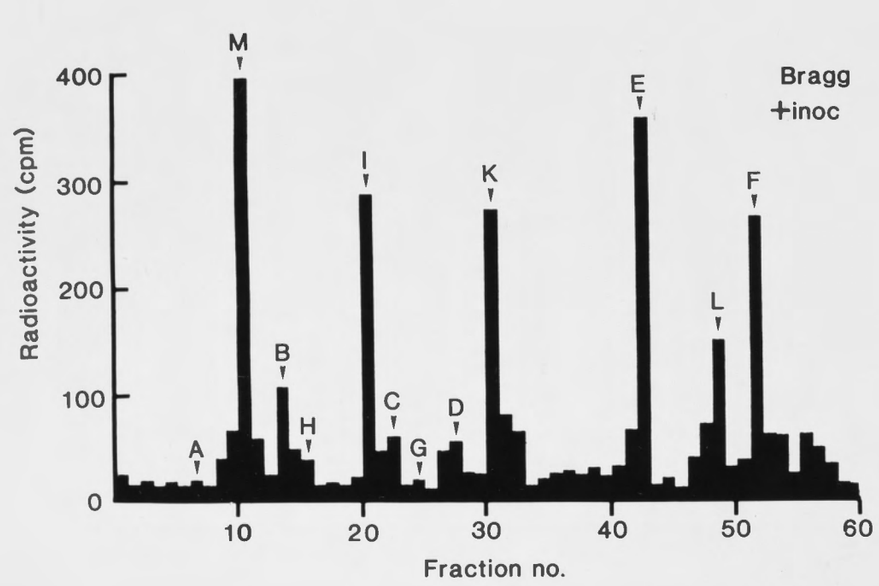
Bragg
+inoc
Radioactivity (cpm)
Fraction no.
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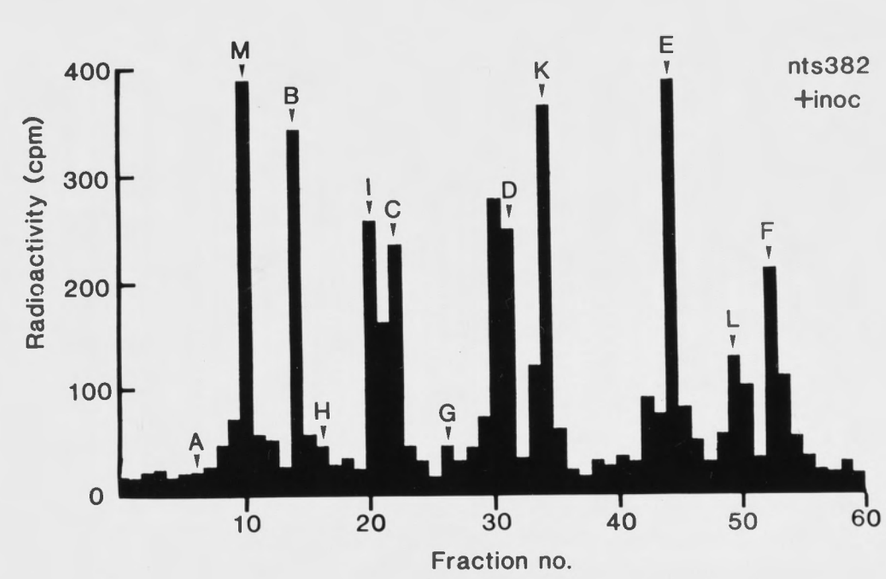
nts382
+inoc
Radioactivity (cpm)
Fraction no.
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CHAPTER FIVE

HOST GENETIC CONTROL OF SOYBEAN NODULATION IN SPLIT ROOT SYSTEMS

5.1 INTRODUCTION

The production of nitrogen fixing nodules on the roots of legumes after infection by *Bradyrhizobium* is an energy expensive process which is controlled via the process, referred to by Carroll *et al.* (1985a), as autoregulation. Pierce and Bauer (1983) showed that this occurs on the tap root of wild-type soybean, such that there is developmental suppression of further nodules by previously induced infections (see also Ridge and Rolfe 1986).

Suppression of nodulation has also been observed in split root systems in which inoculation of second side was suppressed by a prior inoculation of the first side (Singleton 1983; Kosslak and Bohlool 1984; Singleton and van Kessel 1987; Sargent *et al.* 1987). This chapter attempts to clarify the connection between the autoregulation response observed on tap roots and the nodule suppression demonstrated in split root systems.

The suppression of nodule development in split root systems was first observed by Singleton (1983), in an investigation of the effects of salinity on the components o the soybean - *Rhizobium* symbiosis. He observed that suppression of nodulation on the second side was caused by a 48 hour prior inoculation of the other, separately cultured half root.

This observation was followed up by Kosslak and Bohlool (1984), in an investigation of the host's involvement in the control of nodule development. They demonstrated that in a split root system of soybean (*G. max* cv. Lee) inoculation of one half side suppressed subsequent development of nodulation on the opposite side. At zero time, the first side was inoculated with *R.japonicum* strain USDA138 as the primary inoculum. In a short day season, nodulation of the second side was significantly suppressed when the secondary inoculum was delayed for 96 hours. 100% suppression was observed when inoculation of the second side was delayed for 10 days.

In a long day season, inoculation of the second side was highly significant, but not always 100%. They suggested that suppression was due to the photosynthetic potential of the host. Further to this, Bohlool *et al.*(1986) showed the suppression effect (*G. max* cv. Davis) was more pronounced in winter when dry matter production was lower. However, their explanation was inadequate to explain the observed suppression, and made no mention of the involvement of an autoregulatory system.

Singleton and van Kessel (1987) used a split root system (*G. max* cv. Davis) designed to maintain control of the root atmospheres. They wanted to ascertain whether the root portion that was involved in nitrogen fixation received more of the translocated

photosynthate. Their results indicated that (a) dry matter and current photosynthate (^{14}C) were selectively partitioned to nodules and roots where N_2 was available and (b) the flux of current photosynthate to N_2 fixing nodules and their associated roots was greater than in non N_2 fixing roots. It was concluded that the control of carbon flow to roots and nodules was affected by the output of N fixation production from nodules. Their results also showed that if an un-inoculated root system was separately offered ammonium nitrate,* that the photosynthate was preferentially translocated to the side utilizing the nitrate.

In a similarly designed experiment, Sargent *et al.* (1987), demonstrated the suppression of nodulation in in split root systems of clover (*Trifolium subteraneum*). They showed that a 24 hour prior inoculation of one side suppressed subsequent nodulation on the other side, and attributed this to the involvement of a systemic, rapid feedback mechanism in the host which prevents excessive nodulation.

In all of these split root system, plants were grown in the absence of nitrate in the nutrient solution (apart from the ammonium nitrate experiment * above). In addition it was observed that the first inoculated side, (or the side where N_2 was available) developed a larger root system. Thus it is conceivable that the suppression of nodulation observed was due to the roots of the first side responding to the inoculant and producing nodules, but depriving the second side of nitrate assimilates required for growth, thereby inhibiting nodulation. This is supported from data by Singleton and van Kessel (1987).

This chapter included a repeat of these split root experiments, but in addition, provided plants with a small non-inhibitory level of nitrate, so that the physiological functionality of both root portions was assured. In addition, the nitrate tolerant, mutant nts382 (Carroll *et al.* 1985 a,b) was used, since it had previously been shown that the recessive mutation lead to an apparent absence of autoregulation, through the loss of an inhibitory signal from the shoot (Delves *et al.* 1986, Gresshoff and Delves 1986). Thus the use of the nts382 mutant in comparison with its wild-type parent cultivar Bragg provided the opportunity to test whether the suppression of nodulation in split root systems is indeed due to autoregulation, acting in a systemic fashion throughout the entire plant.

In addition the following questions were asked:

1. How do Bragg and nts382 respond to delayed inoculations in split root systems?
2. Are the nodule responses in (1) altered by nitrate concentration in the nutrient solution ?
3. Does nitrate concentration affect root weight partitioning in split root systems ?

4. What is the time course of nodule suppression on the second side of a split root system (*G. max* cv. Bragg) caused by a prior inoculation of the first side ? Does the time course of nodule suppression approach that seen in previous single tap root and split root experiments ?
5. In split root systems, is root fresh matter selectively partitioned to the half root side (and subsequently nodules) which was inoculated first ?
6. Is ^{14}C-sucrose preferentially translocated to the root and nodules of the first inoculated side?
7. Do different soybean genotypes display different autoregulation capacities ?

5.2 MATERIALS AND METHODS

5.2.1 *RHIZOBIUM* STRAINS AND CULTURE

Bradyrhizobium japonicum strain USDA110 was used throughout this study. It was originally supplied by Dr John Streeter (Ohio Res. and Ser. Centre, Wooster USA).

5.2.2 PLANT MATERIAL

The two lines of soybean (*Glycine max* (L.) Merr) used consistently throughout this study were the wild-type cultivar Bragg (from D. Herridge, NSW Department of Agriculture, Tamworth, NSW and H. Keyser, USDA) and its nitrate tolerant, super-nodulating mutant nts382. The nts1007 mutant and the intermediate nodulator nts1116 were isolated by Carroll *et al.* (1985 a,b). Two wild-type cultivars of soybean, other than Bragg were used in part of this study, namely Williams and Clark. Bragg was derived of germ-plasm leading back to Peking accession, while Clark and Williams were derived of the Mandarin accession. Both gene-pools are quite distinct (Allen and Bhardwaj 1987).

5.2.3 PREPARATION OF PLANT MATERIAL FOR SPLIT ROOT ASSAYS

5.2.3.1 Seed surface sterilization and germination

Seeds were selected for uniformity of size and rinsed with 70 % ethanol for two minutes to kill *Rhizobium* contaminants. The ethanol was rinsed off with two washings of sterile distilled water and the seeds covered with 3 % sodium hypochlorite solution and swirled in this for three minutes. The hypochlorite was drained off and the seeds were washed thoroughly with up to 10 washings of sterile distilled water.

Seeds with damaged endosperm swelled up and the embryo cotyledons became separated and as such were discarded. Intact seeds were transferred (15 / plate) to water agar plates (12 g agar / litre distilled water). Molten agar was dripped on each seed to keep it in place. The petri dishes were sealed with Nescofilm and an air-hole (0.5 cm) was made to facilitate gas exchange. Plates were left at an angle of 45º in the dark for two days.

5.2.3.2 Preparation of planting elbows and generation of split roots

The split root planting elbows (electrician's inspection elbow) were prepared by drilling a 10 mm planting hole at the top of the bend. The hole was covered with tape, and elbow was filled with fine grade (No 3) vermiculte and both ends were sealed with suba seals (No.33) and autoclaved. Prior to planting, each elbow was moistened with 10 ml of one quarter strength Herridge solution containing 0.5 mM KNO_3.

When the roots were approximately 7 - 10 cm long, 2mm of the root apical tissue was removed with a scalpel blade to ensure lateral root development and each seedling was transferred to a split root elbow. One seedling was planted per elbow making sure that capillarity was maintained between the cut surface of the root and the vermiculite. The suba seals were removed and the lower 3 cm of each split root elbow was "planted" in a pre-moistened tray of vermiculite. The purpose of using the elbow was to guide the lateral roots in opposite directions so that they could be separated into two separate growth chambers (i.e. split root tubes) for use in a variety of autoregulation studies.

Each tray of elbows was placed under a plastic canopy (to prevent contamination by *Rhizobium*) in a naturally lit glasshouse containing supplementary light sources providing a photon irradiance of 650 $\mu E\ m^{-2}\ sec^{-1}$ for a 16 hour photoperiod. Day / night temperatures were kept consistently between 19 and 30ºC. The plant cultures were irrigated daily with quarter strength Herridge solution containing 0.5 mm KNO_3.

After 5-7 days under these conditions, lateral roots had usually emerged from both sides of the elbow, and only those that had equal lateral root development on either side (approximately 90 %) were used.

The final stage of the split root assembly involved attaching a PVC tube (25 cm length, 42 mm o.d., 37 mm i.d., SWV pipe, Iplex plastics), filled with pre-moistened sterile vermiculite (90 ml I/4 strength Herridge + 0.5 mM KNO_3) to either side of the elbow, so as to provide a separate growth chamber for each half side of the split root system. The ends of each column were sealed with a double layer of Nescofilm, held in place with a

PVC joiner (40 mm i.d., 60 mm length) The complete apparatus was left in the conservatory for 24 hours prior to inoculation (see figs. 5.1 and 5.2).

5.2.3.3 Inoculation of the split root apparatus

Each tube was inoculated by injecting 5 ml (approx. 10^9 cells) of a peat inoculum slurry (see section 2.4.2) (with a pasteur pipette) through the inoculation/ watering port. Using this method it was possible to prevent transfer of *Rhizobium* from one side to the other. Sides not receiving inoculum were injected with 5 ml of sterile nutrient solution.

The second inoculation (D = Delayed inoculation) occurred either 24 h, 48 hr, 4 or 7 days after inoculation of the first side, following the same procedure.

5.2.3.4 Subsequent watering of the split root apparatus

Each leg was watered separately (rather than via the planting hole which could lead to (a) contamination from one side to the other and (b) uneven distribution of nutrient solution) with Herridge nutrient solution (supplemented with 0.5 mM KNO_3) every five days, using one-quarter strength nutrients for the first watering and full strength thereafter. A drainage hole at the bottom of each leg facilitated drainage, and the Nescofilm at the bottom each tube facilitated gas exchange.

5.2.3.5 Harvesting of split root apparatus

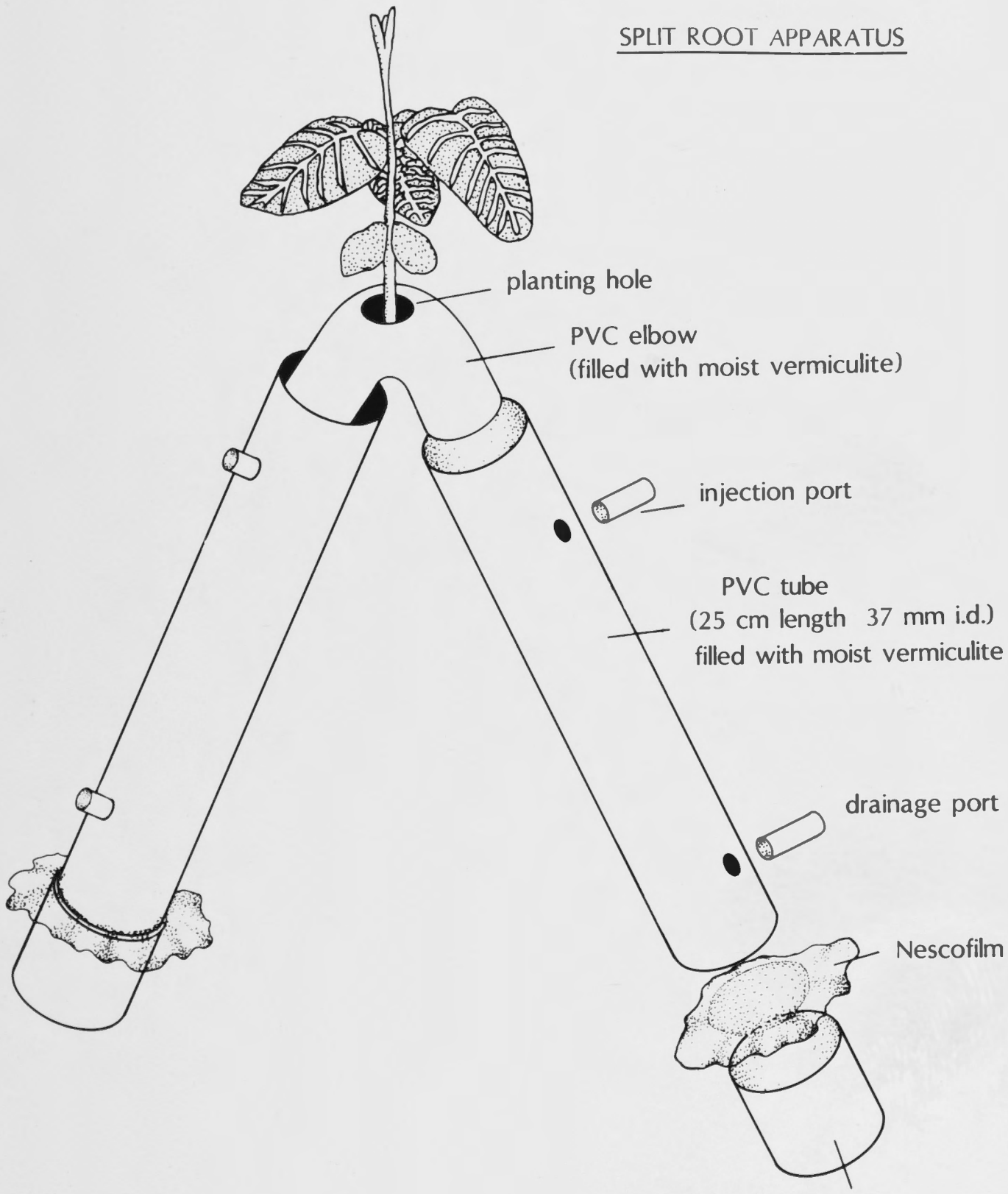
The PVC joiners and Nescofilm were removed and the vermiculite shaken out of the legs. After this the elbow was gently removed (by cutting away the Nescofilm), taking care not to damage the root systems. The vermiculite was washed and pulled off and in the majority of cases, the intact root system could be pulled out through the planting hole.

Various measurements such as shoot weight, stem length, root weights, nodule numbers, nodule weights and nodulation profile were routinely taken and other measurements were made as experimental design decreed.

Experiment 12(a) was performed in collaboration with Dr. B. Bohlool at the University of Hawaii, and used a similar split root system to that described above, with variations being made to suit local supplies. A description of this set-up can be found in the materials and methods section of a combined publication (Olsson *et al.* 1988).

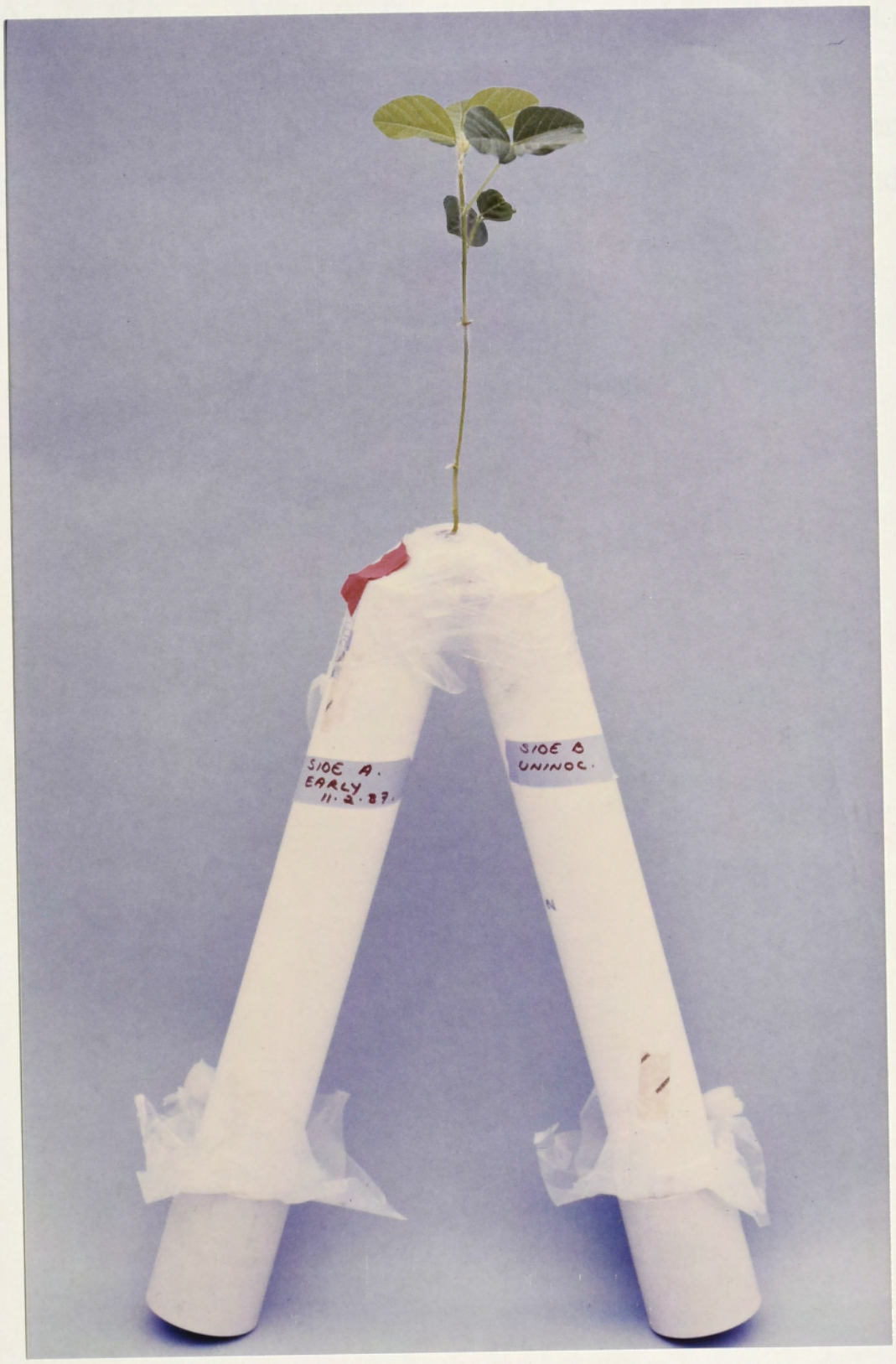
Figure 5.1 Split root apparatus - exploded diagram

SPLIT ROOT APPARATUS



PVC joiner (40 mm i.d. 60 mm length)

Figure 5.2 Photograph of a Bragg plant in a split root apparatus


SIDE A.
EARLY
11-2-87.
SIDE B
UNINOC.

5.2.4 PREPARATION OF MATERIAL FOR LIQUID SCINTILLATION COUNTING

Split roots were harvested as described above. Nodules of each half root were detached, weighed and transferred to a PVC vial. The corresponding root was cut into 3 cm lengths, weighed, and each was transferred to a separate vial. Samples were left overnight in 10 ml of scintillation mixture (see section 2.6.1). Activity was detected on a Beckman LS 7000 scintillation counter. Radioactivity in either half root, or in the nodules of either half root, was expressed as a percentage of the total amount in the whole root plus nodules. The specific root radioactivity of each half system was derived from the total cpm per gram of fresh root weight. Similarly the specific nodule radioactivity was derived from the total cpm per nodule.

EXPERIMENTAL DESIGN AND RESULTS

5.3 EXPERIMENT 12(A): INVESTIGATION OF THE AUTO - REGULATION CAPACITY AND ROOT WEIGHT PARTITIONING OF CV.BRAGG AND NTS382 GROWN IN THE ABSENCE OF NITRATE.

5.3.1 DESIGN

Split roots of cv. Bragg and nts382 were set up as described in section 5.2.3 and grown in a N-free nutrient solution (as described in Kosslak and Bohlool 1984) to examine the effect of delayed inoculation on nodule suppression and root growth as compared to a low level nitrate environment (see (b) below). The experiment also looked at the compensation of nodule numbers in doubly and singly inoculated plants.

Split roots systems were inoculated in the following patterns:

	SIDE 1	SIDE 2
(a)	T = 0 h (Early)	Un-inoculated
(b)	T = 0 h (Early)	T= 0 h (Early)
(c)	T = 0 h (Early)	T = 7 d (Delayed)
(d)	T = 7 d (Delayed)	T = 7 d (Delayed)
(e)	Un-inoculated	T = 7 d (Delayed)

5.3.2 Results

Bragg minus nitrate (Early / Early)

Table 5.1 examines the effect of delayed inoculation on the resultant nodule numbers in split root systems grown in the absence of nitrate. When both sides of the Bragg split root system were inoculated at T = 0 h (Early / Early), equal numbers of nodules developed on either side (i.e. 23 ± 9 and 26 ± 12) and the two half roots demonstrated no significant difference in rootbiomass production (i.e. 106 ± 27 mg and 95 ± 40 mg).

Bragg minus nitrate (Early / Delayed)

When inoculation of the second side was delayed until 7 days after the first side (Early / Delayed pattern), 100 % nodule suppression was observed on the delayed side (i.e. side 1 = 53 ± 13 nodules; side 2 = 0 ± 0 nodules). This total suppression of nodulation was accompanied by a corresponding significant suppression of root dry weight on the delayed side (i.e. 119 ± 32 mg and 70 ± 7 mg respectively).

Table 5.1: Nodulation and root growth of split root grown cv. Bragg and nts382 in the absence of external nitrate (Experiment 12a)

	SIDE	INOCULATION PATTERN	NN	NODULE (mg)	RDW (mg)	% Suppression
Bragg	1	T= 0 hrs (Early)	53 ± 7	59 ± 11	123 ± 33	N/A
	2	Un-inoculated	0 ± 0	0 ± 0	86 ± 21	
	1	T = 0 hrs (Early)	23 ± 9	31 ± 10	106 ± 27	N/A
	2	T = 0 hrs (Early)	26 ± 12	34 ± 13	95 ± 40	
	1	T = 0 hrs (Early)	53 ± 13	49 ± 8	119 ± 32	100
	2	T = 7 days (Delayed)	0 ± 0	0 ± 0	70 ± 7	
	1	T = 7 days (Delayed)	44 ± 12	20 ± 4	78 ± 16	N/A
	2	T = 7 days (Delayed)	60 ± 10	24 ± 6	83 ± 9	
	1	Un-inoculated	0 ± 0	0 ± 0	49 ± 13	N/A
	2	T = 7 days (Delayed)	120 ± 32	42 ± 10	104 ± 15	
		LSD 0.05		19	14	
nts382	1	T = 0 hrs (Early)	268 ± 53	126 ± 15	56 ± 17	N/A
	2	Un-inoculated	0 ± 0	0 ± 0	54 ± 23	
	1	T = 0 hrs (Early)	230 ± 109	66 ± 30	48 ± 29	N/A
	2	T = 0 hrs (Early)	269 ± 150	77 ± 23	55 ± 18	
	1	T = 0 hrs (Early)	253 ± 63	110 ± 26	67 ± 29	78
	2	T = 7 days (Delayed)	56 ± 36	7 ± 3	60 ± 28	
	1	T = 7 days (Delayed)	260 ± 85	51 ± 20	76 ± 22	N/A
	2	T = 7 days (Delayed)	192 ± 62	40 ± 13	58 ± 18	
	1	Un-inoculated	0 ± 0	0 ± 0	72 ± 17	N/A
	2	T = 7 days (Delayed)	342 ± 10	89 ± 6	67 ± 17	
		LSD 0.05	110	25	9	

All data expressed as $\bar{x} \pm$ s.d; NN = nodule number per half root system; RDW = root dry weight per half root system; N/A = not applicable.

Bragg minus nitrate (Delayed / Delayed)

When Bragg plants were synchronously inoculated at T = 7 days (Delayed / Delayed), there was no significant difference in nodule number on either side (i.e. 44 ± 12 and 60 ± 10 nodules respectively, nor was there any difference in root weight partitioning to either side (i.e. 78 ± 16 and 83 ± 9 mg respectively).

Table 5.1 demonstrates that Bragg split root systems, grown in the absence of nitrate exhibited nodule number compensation. In an Early / Un-inoculated system, the total number of nodules was 53. When both sides were inoculated at T = 0 h (Early / Early), the total nodule number was 49, and in an Early / Delayed system, the total nodule number was 53.

Similarly, in Delayed / Delayed systems the total nodule number was 104, and in Un-inoculated / Delayed system, the total nodule number was 120. The increase in nodule number in the latter two systems indicates that the plant will produce more nodules if its root system is allowed to develop for one week prior to inoculation.

In all systems however, Bragg plants displayed nodule weight compensation (see table 5.1), with approximately 42 to 65 mg nodule dry weight being produced.

Nodule dry weight was reduced in Delayed / Delayed systems presumably because of the reduced time (by one week) for nodule growth. Root dry weights of Early / Early and Delayed / Delayed inoculated plants were similar, (being in contrast to results from Table 5.2, which showed an advantage of delayed inoculation on root growth). Un-inoculated root halves did not grow as well as inoculated root halves, fitting into the suggestions put forward by Singleton and van Kessel (1987) regarding preferential allocation of photosynthate to nodulated roots.

Nts382 minus nitrate (Early / Early)

When both sides of an nts382 split root system were inoculated at T = 0 h (Early / Early), no significant difference was observed between the nodule number of side 1 and side 2 (230 ± 109 and 269 ± 150 nodules respectively).

Nts382 minus nitrate (Early / Delayed)

When side 2 inoculation was delayed for 7 days (Early / Delayed), there was 78 % suppression of nodulation on that side compared to side 1, however in contrast to similarly treated Bragg plants, there was no suppression of side 2 root biomass producton on side 2 (i.e. 67 ± 29 mg and 60 ± 28 mg respectively).

Nts382 minus nitrate (Delayed / Delayed)

When nts382 split roots were both inoculated at T = 7 days (Delayed / Delayed), the number of nodules which formed were 260 ± 85 and 192 ± 62 respectively. There represented no significant difference in nodule number.

These data demonstrate that in the absence of nitrate in the nutrient solution, that a significant suppression of nodule number was observed on both Bragg and nts382 roots as a result of a 7 day delay in inoculation on one root half. This was accompanied by a significant suppression in Bragg, but not nts382 root dry weight.

Comparison of an Early / Early inoculated set of nts382 plants with an Early / Un-inoculated set (see table 5.1) showed that nodule number compensation did not occur, with the Early / Early treatments resulting in twice the nodule number per plant. Nodule dry weight however was compensated with 126 ± 15 mg dry root wt. being similar to 143 ± 30 mg. The lowered nodule mass on the Un-inoculated / Delayed plants (i.e. 89 mg) was again the result of a reduced time to complete nodule growth as compared to the Early / Un-inoculated control (i.e. 126 mg). Numbers of nodules per half root system in the Early / Delayed inoculated set was indicative of some suppression of nodulation. Although nodule number and nodule mass could be slightly "discounted" on the delayed side for the decreased time available to develop, it was still clear that suppression of nodulation occurred. Moreover, the effect of suppression in the Early / Delayed set of plants of nts382 was more pronounced when the nodule dry weight was compared (100 ± 26 mg vs. 7 ± 3 mg).

Root dry weights in nitrate free split root systems of Bragg tended to show that the first inoculated root was larger. This was not seen for nts382 plants treated similarly.

Although there was a slight trend for nitrate treated Bragg plants to selectively develop root fresh weight on the side of the split root system that was first inoculated, the differences were not statistically significant at the 1% confidence level. Moreover, no significant difference was observed in fresh weights of split root systems of Early / Un-inoculated roots. Similarly, no significant difference was noted between the roots of Un-inoculated / Delayed roots.

5.4 EXPERIMENT 12(B): INVESTIGATION OF THE AUTO - REGULATION AND ROOT WEIGHT PARTITIONING CAPACITY OF CV. BRAGG AND NTS382 GROWN IN THE PRESENCE OF 0.5 MM KNO_3.

5.4.1 DESIGN

Split root systems of the cultivar Bragg and nts382 were set up to investigate their respective autoregulation capacities using *B. japonicum* strain USDA110 as the inoculant, when 0.5 mM KNO_3 was included in the nutrient solution. Three inoculation patterns were used: Both sides were inoculated at T=0 h (i.e. Early / Early); Side 1 was inoculated at T=0 h and side 2 was inoculated at T=7 days (i.e. Early / Delayed); or both sides were inoculated at T=7 days (i.e. Delayed / Delayed) (see Table 5.1, Fig 5.3). Split root assays were prepared as described in section 5.2.3.

5.4.2 RESULTS

Bragg plus nitrate (Early / Early)

Figure 5.3 shows that in control experiments, when both sides of a Bragg split root system were synchronously inoculated at T = 0 h, (i.e. Early / Early) there was no significant difference in the nodule number on either side (i.e. 19 ± 5 and 13 ± 4 respectively). Additionally, there was no significant difference in the root fresh weights on either side 1 or side 2 (i.e. 1.31 ± 0.33 g and 1.47 ± 0.31 g respectively, see table 5.2).

Bragg plus nitrate (Early / Delayed)

When inoculation of the second side was delayed for 7 days (Early / Delayed), nodulation on that side was totally suppressed, (i.e. 31 ± 3; side 1 = 0.3 ± 0.5; see fig 5.3). This suppression of nodulation was not accompanied by a reduction in root fresh weight. Indeed there was no significant difference in root fresh weights between side 1 and side 2 (i.e. 2.12 ± 0.25 g and 1.99 ± 0.28 g respectively; see table 5.2).

Bragg plus nitrate (Delayed / Delayed)

When inoculation of both sides of the split root system were synchronously delayed until day 7 (Delayed / Delayed) there was no significant difference in nodule number (i.e. 34 ± 18 and 30 ± 14 respectively), or root weight partitioning (i.e. 2.04 ± 0.60 g and 2.10 ± 0.50 g respectively, see table 5.2). Synchronous inoculation at T = 7 days substantially increased nodule number per plant compared to synchronous inoculations at T = 0 hours.

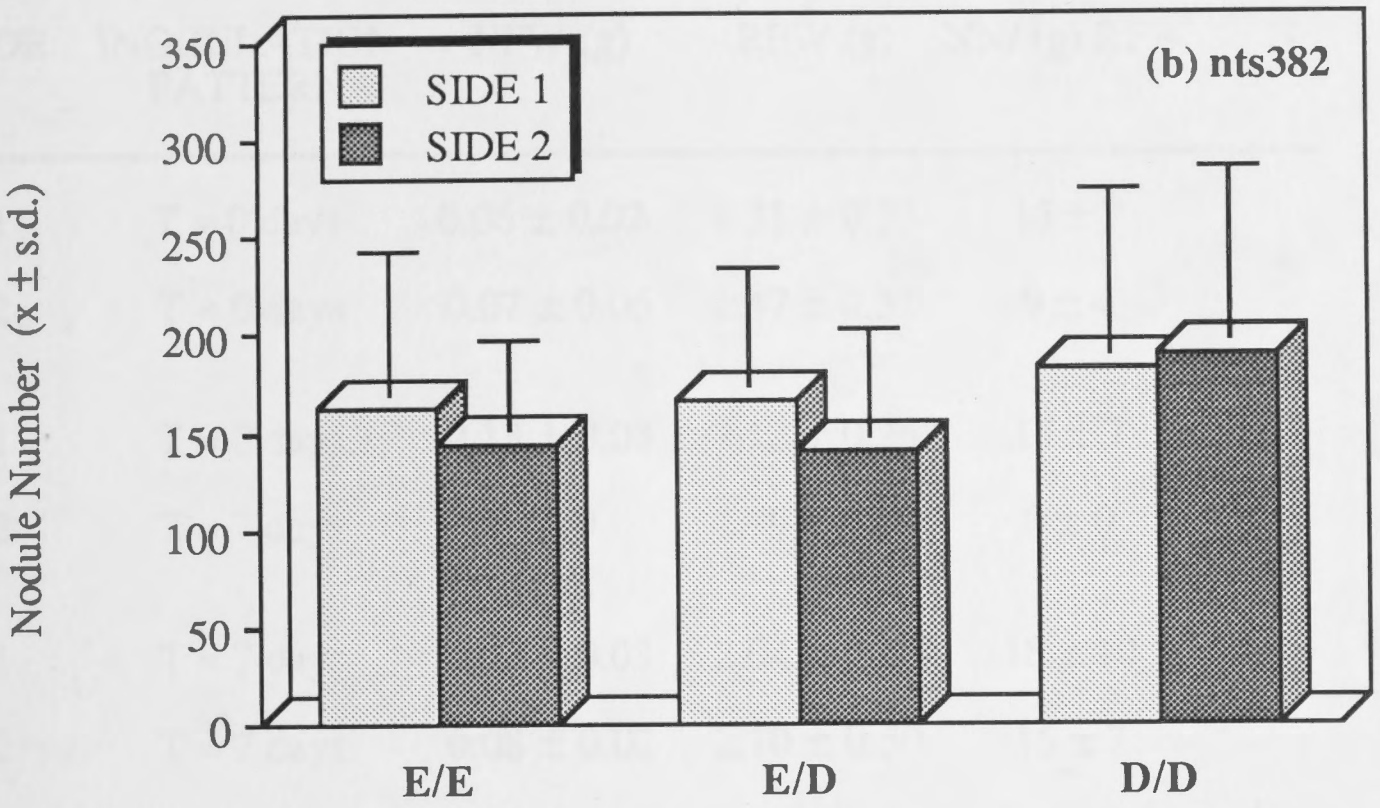
Figure 5.3 **Demonstration of nodule autoregulation in Bragg, (a), and the the absence of nodule autoregulation in nts382, (b) on side 2 of a split root system as a result of a 7 day prior inoculation on the first side.**

E / E = Side 1 inoculated at T = 0 hours; Side 2 inoculated at T = 0 h

E / D = Side 1 inoculated at T = 0 hours; Side 2 inoculated 7 days later (T = 7 days)

D / D = Side 1 inoculated at T = 7 days; Side 2 inoculated at T = 7 days.

Plants were inoculated with *Bradyrhizobium japonicum* strain USDA110


(b) nts382
SIDE 1
SIDE 2
Nodule Number (x ± s.d.)
350
300
250
200
150
100
50
0
E/E
E/D
D/D

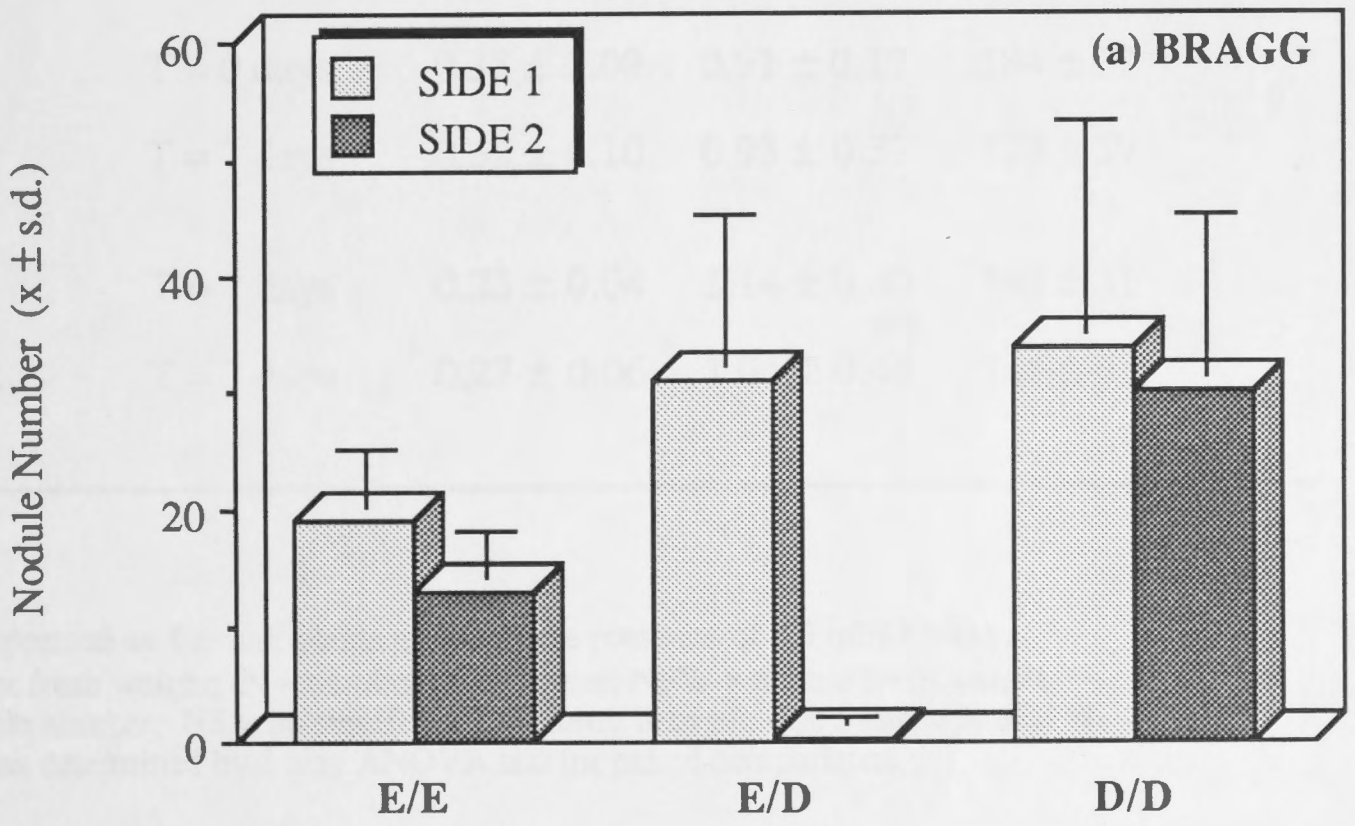

(a) BRAGG
SIDE 1
SIDE 2
Nodule Number (x ± s.d.)
60
40
20
0
E/E
E/D
D/D

Table 5.2: Nodulation and root growth of split root grown cv. Bragg and nts382 in the presence of low level nitrate (Experiment 12b)

	SIDE	INOCULATION PATTERN	NFW (g)	RFW (g)	NN/ (g) RFW	N
Bragg	1	T = 0 days	0.06 ± 0.02	1.31 ± 0.33	15 ± 7	4
				NS		
	2	T = 0 days	0.07 ± 0.06	1.47 ± 0.31	9 ± 4	
	1	T = 0 days	0.13 ± 0.03	2.12 ± 0.25	15 ± 7	5
				NS		
	2	T = 7 days	0 ± 0	1.99 ± 0.28	0 ± 0	
	1	T = 7 days	0.08 ± 0.03	2.04 ± 0.60	18 ± 10	7
				NS		
	2	T = 7 days	0.08 ± 0.02	2.10 ± 0.50	15 ± 7	
nts382	1	T = 0 days	0.30 ± 0.12	0.57 ± 0.28	307 ± 93	4
				NS		
	2	T = 0 days	0.28 ± 0.14	0.56 ± 0.38	308 ± 97	
	1	T = 0 days	0.38 ± 0.09	0.91 ± 0.17	184 ± 67	8
				NS		
	2	T = 7 days	0.32 ± 0.10	0.93 ± 0.37	133 ± 39	
	1	T = 7 days	0.23 ± 0.04	1.14 ± 0.40	144 ± 31	3
				NS		
	2	T = 7 days	0.27 ± 0.06	1.04 ± 0.46	145 ± 63	

All data expressed as $\bar{x} \pm$ s.d; plants grown in the presence of 0.5 mM KNO_3 ;
RFW = root fresh weight; N = number of replicates; NFW = nodule fresh weight,
NN = nodule number; NS = no significant difference between side 1 and side 2 RFW
(P = 0.01) as determined by 1 way ANOVA test for paired comparisons.

Nts382 plus nitrate (Early / Early)

Table 5.2 shows that when both sides of an nts382 split root system were inoculated at T = 0 h, there was no significant difference in nodule number (i.e. 162 ± 73 and 144 ± 46 respectively), and no suppression of root fresh weight (i.e. 0.57 ± 0.28 g and 0.56 ± 0.38 g respectively).

Nts382 plus nitrate (Early / Delayed)

In contrast to Bragg, nts382 Early /Delayed split root systems, grown in the presence of 0.5 mM KNO_3 did not result in a significant difference in nodulation on the delayed side (i.e. 165 ± 61 and 140 ± 55 nodules respectively, see table 5.2). Further, there was no significant difference in root fresh weights (i.e. 0.91 ± 0.17 g and 0.93 ± 0.37 g respectively). The 7 day delay had no effect on nodule fresh weight. Indeed, the total nodule fresh weight from an Early / Delayed system could be summed to equal that on an Early / Early system.

Nts382 plus nitrate (Delayed / Delayed)

Synchronous inoculations at T = 7 days resulted in equal development of nodule number and nodule fresh weight and root fresh weights on either side of the split root system. These three parameters developed equally compared to those seen on Early / Early split root systems. However, Early / Delayed and Delayed / Delayed systems developed larger roots than Early / Early systems.

A comparison of root fresh weights supported the finding (Table 5.2) that nodulated nts382 has smaller roots than cv. Bragg even in the absence of supplied nitrate, thus confirming previous data (Carroll *et al.* 1985a; Day *et al.* 1986). It is also noteworthy to observe a trend for both nts382 and Bragg which showed that delayed inoculation (either one half or the whole of the root system), resulted in a larger root system. This was more pronounced in nts382, again corroborating data presented by Day *et al.* (1986) in which they showed that non-nodulated, but nitrate grown, Bragg and nts382 plants grew better than inoculated controls.

Further, whereas un-inoculated versus inoculated (either early or delayed) sets of cv. Bragg plants gave different sized roots, the same treatments caused similar sized roots in nts382 plants. The nature of the interaction between nodulation and root growth is at present not understood and may involve multiple effects such as source/sink relations and microbial stimulation of root growth.

5.5 EXPERIMENT 13: INVESTIGATION OF THE TIME COURSE OF AUTOREGULATION IN CV. BRAGG SPLIT ROOT SYSTEMS.

5.5.1 DESIGN

Split root systems of cv. Bragg were set up to examine the time course of nodule autoregulation. Side 1 was inoculated with *B. japonicum* strain USDA110 at time zero (T=0). Inoculation of the second side was delayed for 0, 24 hrs, 48 hrs, 4 days or 7 days after inoculation of the first side. Control experiments involved either inoculation of side 1 at T=0 and leaving side 2 un-inoculated, or leaving side 1 un-inoculated and delaying the inoculation of side 2 for 7 days.

Plants were grown for 3 further weeks after the inoculation of side 2. Split root systems set up was as described in section 5.2.3. All plants were watered with Herridge solution supplemented with 0.5 mM KNO_3, as the previous experiment (1b) demonstrated the requirement for a low level of nitrate in the nutrient solution.

5.5.2 RESULTS

Table 5.3 looked in more detail at the time required for the delay to achieve suppression of nodulation. As additional controls half inoculated root portions were also included. Again the phenomenon of nodule number compensation was notable as Early / Un-inoculated plants had 23 ± 11 nodules on the inoculated side, while Early / Early plants had an equal number of nodules per side, summing up to 23. Nodule mass followed a similar trend. Already, after 24 hours delay of second inoculation, one observed a suppression of around 70%. This level of suppression was maintained for the first 4 days of inoculum delay and rose to 100% suppression, resulting from a 7 day delay. These findings supported those seen in fig 5.3, and indicate that suppression of nodulation is a rapid phenomenon. The root fresh weight data for this experiment is incorporated in Table 5.4 and will be discussed in the next section (5.6).

Table 5.3: Time course of nodule suppression in a split root system of *G.max* cv. Bragg (Experiment 13)

SIDE	INOCULATION PATTERN	N	NODULE NO.	% NODULE SUPPRESSION	NFW (mg)
1	T = 0 Days	5	23 ± 11 S*		70 ± 20
2	Un-inoculated		0	N.A.	0
1	T = 0 days	5	13 ± 4 NS*		56 ± 20
2	T = 0 days		10 ± 4	13	40 ± 20
1	T = 0 days	4	18 ± 14 S**		70 ± 70
2	T = 24 hours		4 ± 3	55	40 ± 30
1	T = 0 days	6	17 ± 15 S**		50 ± 30
2	T = 48 hours		7 ± 6	76	40 ± 40
1	T = 0 days	7	26 ± 15 S*	88	70 ± 60
2	T = 4 days		3 ± 3		40 ± 40
1	T = 0 days	9	17 ± 8 S*	100	80 ± 70
2	T = 7 days		0		0
1	Un-inoculated	4	0 S*	N/A	0
2	T = 7 days		18 ± 8		9

All data expressed as $\bar{x} \pm$ s.d.; all tests were run in the presence of 0.5mM KNO_3
N.A. = Not applicable; N = number of replicates; NFW = nodule fresh weight;
% Nodule Suppression = Reduction in % nodulation as compared to its respective T = 0 pair.
For root fresh weights see Table 5.4;
NS* = no significant difference between side 1 and side 2 (P = 0.01);
S** = significant difference between side 1 and side 2 (P = 0.05);
S* = significant difference between side 1 and side 2 (P = 0.01) as determined by 1 way ANOVA test for paired comparisons.

5.6 EXPERIMENT 14: INVESTIGATION OF THE PARTITIONING OF ^{14}C - SUCROSE TO THE ROOTS AND NODULES OF CV. BRAGG SPLIT ROOT SYSTEMS.

5.6.1 DESIGN

Plants in experiment 13 were also examined for photosynthate partitioning in split root systems. The shoot of each plant was injected with 5 µl ^{14}C-sucrose (radioactive concentration = 1.48 kBq/l; specific activity = 20.7 GBq/mMole) 24 hours prior to harvest when the nodules were detached and the radioactivity (cpm) of both nodules and roots of each half system was measured. The radioactivity in the roots or nodules of either side was expressed as a percentage of the total counts of the roots plus nodules of both sides. Each half root system (minus nodules) was also weighed immediately after harvest to see if fresh root matter was preferentially partitioned.

5.6.2 RESULTS

Whilst a slight (unexplainable) preferential allocation of root fresh or dry weight to the first inoculated side was noted (see above), no difference in the allocation of injected ^{14}C-sucrose to each half root system was detected (Table 5.4). This inferred that at the time of harvest (3 weeks after inoculation of the delayed side) under all inoculation patterns, there was equal distribution of the ^{14}C-sucrose to each half root system.

When both sides of cv. Bragg split root system were inoculated at T=0 h (Early/Early) ^{14}C-sucrose was partitioned equally to nodules of either side. However a prior inoculation of 24 h, 48 h, 4 days or 7 days on one side of the split root system resulted in a greater percentage of the total ^{14}C (as cpm) to be translocated to the nodules of the first inoculated side. In systems where inoculation of the second side was delayed for 7 days, there was a total suppression of nodulation on that side and hence no ^{14}C was partitioned to the nodules. However when specific nodule radioactivity (i.e. cpm per nodule) was calculated there was no significant difference between that seen in the nodules resulting from an early as opposed to a delayed inoculum. Hence although suppression occurred, the resultant nodules were equally functional in their ability to attract injected ^{14}C-sucrose.

Table 5.4: Partitioning of ^{14}C-sucrose to nodules and roots of *G. max*. cv. Bragg (Experiment 14)

SIDE	INOCULATION PATTERN	N	Radioactivity in nodules (% of total in roots)	Radioactivity in roots (% of total in roots)	Root fresh wt. (g)
1	T = 0 hrs	5	11 ± 1	50 ± 5	0.86 ± 0.19
2	Un-inoculated		0 ± 0	40 ± 2	NS 0.68 ±0.12
1	T = 0 hrs	5	8 ± 2	45 ± 4	0.99 ± 0.22
2	T = 0 hrs		8 ± 1	38 ± 3	NS 0.93 ± 0.33
1	T = 0 hrs	4	12 ± 6	38 ± 20	0.79 ± 0.60
2	T = 24 hrs		13 ± 13	38 ±16	NS 0.55 ± 0.23
1	T = 0 hrs	6	11 ± 2	38 ± 3	0.99 ± 0.38
2	T = 48 h		8 ± 3	43 ± 3	NS 0.55 ± 0.23
1	T = 0 hrs	7	13 ± 5	43 ± 11	1.17 ± 0.34
2	T = 4 days		5 ± 2	39 ± 11	NS 0.85 ± 0.37
1	T = 0 hrs	9	13 ± 3	41 ± 8	0.94 ± 0.39
2	T = 7 days		0 ± 0	45 ± 10	NS 0.65 ± 0.25
1	Un-inoculated	4	0 ± 0	50 ± 12	0.94 ± 0.39
2	T = 7 days		6 ± 4	44 ± 11	NS 1.15 ± 0.69

All data are expressed as $\bar{x} \pm$ s.d.
N = number of replicates
NS = no significant difference between side 1 and side 2 root weights (as determined by LSD 0.05)
All plants were grown in the presence of 0.5 mM KNO_3.

5.7 EXPERIMENT 15 : INVESTIGATION OF SOYBEAN CULTIVAR VARIABILITY ON AUTOREGULATION.

5.7.1 DESIGN

Split root systems of cv. Bragg, Williams and Clark as well as nts382, nts1007 and the intermediate hyper-nodulating mutant nts1116 (Carroll *et al.* 1985a,b; Delves *et al.* 1986; Gresshoff *et al.* 1986) were used to ascertain the degree of variability within the host plant in the autoregulation response. Side 1 of each system was inoculated at time zero with strain USDA110 and the inoculation of side 2 was delayed for a further 7 days. Plants were irrigated with 1/4 strength Herridge nutrient solution supplemented with 0.5 mM KNO_3 for the first 14 days after germination and thereafter with full strength nutrient solution plus 0.5 mM KNO_3 nitrate. Plants were harvested 17 days after the inoculation of side 2. Growth conditions were as described above.

5.7.2 RESULTS

In Table 5.5, (as shown above), Bragg almost totally suppressed nodulation on the second half of a split root system, after a 7 day prior inoculation of the first side. Mutant nts382 however, did not respond in the same manner as Bragg, giving equal nodulation on both sides. Both cultivar Clark and Williams demonstrated a substantial degree of autoregulation giving suppression of around 70%. Hence both Clark and Williams were designated weaker autoregulators than Bragg. Yet control experiments show that total nodule numbers per plant in cv. Bragg, Williams and Clark were relatively similar, suggesting that the here observed difference may be a result of timing and perhaps nodulation pattern, rather than a direct nodule initiation pattern.

Mutant nts1116 was isolated by Carroll *et al.* (1985 a,b) and was labelled a hyper-nodulation mutant (Gresshoff and Delves 1986), with nodulation being much greater than Bragg but less than nts382 (see Table 1 in Delves *et al.*, 1986). Its pattern of nodule suppression (from 100% to 26%) on the second side of a split root system was intermediate between parent cultivar Bragg and nts382. The hyper-nodulation phenotype was also noticed by the elevated nodule number on side 1 (38 for Bragg versus 105 for nts1116). Nts1116 thus had nodule suppression similar to that of the wild-type cultivars Clark and Williams, yet its nodulation pattern was different.

Mutant nts1007 did not behave as expected in a split root system. When grown as a single plant it demonstrated super-nodulation much like nts382, however in a split root it behaved like nts1116 with nodulation on side 2 being approximately 30% of side 1.

Table 5.5 **Nodulation and root fresh weights (less nodule weight) of various genotypes of soybeans in split root systems. (Experiment 15)**

GENOTYPE	SIDE	NN	% NODULE SUPPRESSION	RFW (g)	% ROOT SUPPRESSION	N
BRAGG	1	38 ± 8	99	1.58 ± 0.57 NS	46	6
	2	0.4 ± 0.8		0.86 ± 0.34		
CLARK	1	25 ± 8	64	1.71 ± 0.55 S	53	7
	2	9 ± 4		0.81 ± 0.40		
WILLIAMS	1	33 ± 12	76	1.45 ± 0.33 S	41	9
	2	8 ± 4		0.86 ± 0.31		
nts1116	1	105 ± 28	70	0.73 ± 0.29 NS	25	4
	2	29 ± 19		0.55 ± 0.19		
nts1007	1	172 ± 54	23	0.54 ± 0.43 NS	26	3
	2	51 ± 23		0.40 ± 0.42		
nts382	1	108 ± 22	78	0.69 ± 0.25 NS	0	6
	2	83 ±18		0.69 ± 0.07		

Side 1 was inoculated at T = 0 hours (Early); Side 2 was inoculated at T = 7 days (Delayed)
Both sides were inoculated with *B. japonicum* strain USDA110;
All data are expressed as $\bar{x} \pm$ s.d.,
N = number of replicates.
S = significant difference between side 1 and side 2 RFW (P = 0.01)
NS = no significant difference between side 1 and side 2 RFW (P = 0.01)
% Suppression = reduction in (a) nodule number or (b) root fresh weight on side 2 compared to side 1.

Nts1007 showed its super-nodulation phenotype by development 172 nodules on the first inoculated root portion. Genetic analysis (Gresshoff and Delves 1986; Delves A.C., pers. comm.) suggests that nts382 and nts1007 are in the same complementation group indicating that alleles have different phenotypes under different assay conditions.

5.8 DISCUSSION

Kosslak and Bohlool (1984), and Sargent *et al.* (1987) implied that the observed nodule suppression, resulting from a 7 day delay in inoculation of one side of a split root system is the result of a host controled systemic response. The inadequacy of their explanations however, was that the first inoculated root was often larger. This was confirmed by results in experiment 12(a) of this chapter and suggested that the observed suppression may have been due to the first inoculated side responding to the inoculum dose, in a similar way to nutrient supply (Drew *et al.* 1973). This would thus deprive the second side of metabolites required for growth, thereby inhibiting nodulation. To counteract this possible effect, a low level of non-inhibitory nitrate (0.5 mM KNO3) was supplied in the nutrient solution, because it is known that roots do respond to nitrate supply. This resulted in total suppression of nodulation on the delayed side of an Early / Delayed system, without any suppression of root weight.

The requirement for a low level of nitrate in the system was further demonstrated with results from nts382 plants. In the absence of nitrate, a 7 day delay in inoculation resulted in 78 % suppression of nodulation on the delayed side of and Early / Delayed system. There was no significant difference in root weights between the two sides. However, in the presence of nitrate, nodule suppression on the second side was corrected.

The most likely explanation for this difference is that when split roots are grown in the absence of a nitrogen source, the amount of "structural resources" available to the second inoculated side becomes a limiting factor to its growth and nodulation potential. For nts382, in the absence of nitrate, if one presumes that the growth of the root has preferential demand for the limiting "structural resources" over the nodules, then nodule suppression can be explained. However if low level nitrate (0.5 mM KNO_3) is applied, then the allocation of these structural resources to both root portions is equal (see also Singleton and van Kessel 1987). Hence the limitation seen in N free plants will not occur and thus the nodulation capacity of nts382 can be completely expressed. That is, the suppression of nodulation observed in Early / Delayed split root systems grown in the absence of nitrate, is caused by a systemic autoregulation factor but is accentuated by the limitation of nitrate, as illustrated by the accompanying reduction in root weight.

. These data demonstrate that care needs to be taken when interpreting data from split root experiments, because the roots can obviously respond to nutrient supply or inoculation by *Bradyrhizobium*. The latter is minimized if a small amount of nitrate is included in the nutrient solution.

Time course of nodule suppression

When both sides of a cv. Bragg split root were inoculated at T= 0 h (Early / Early), nodule number and nodule weights were approximately equal. However when inoculation of the second side was delayed for 24 h or more, it caused a significant suppression of nodule number and nodule fresh weight compared to the zero time treatment. When inoculation of the second side was delayed for 7 days, nodulation on that side was totally suppressed. These data compare to those of Kosslak and Bohlool (1984) who showed that in a short day season, significant suppression of the second side could be observed if the inoculation was delayed for 48 hours and total suppression if inoculation was delayed 10 days.

Suppression of nodulation of the second side resulted from a 24 h (or greater), delayed inoculation. This result is slightly slower than the time scale of autoregulation noted by Pierce and Bauer (1983), who used pouch grown soybeans and double inoculations to propose that the inhibition of further nodulation events was induced within 15 to 17 hours, if nodulation only along the tap root was scored. However, Calvert *et al.*(1984), using serial sections of similarly treated plants suggested that Pierce and Bauers original hypothesis was wrong. Additionally, Takats (1986) and Heron and Pueppke (1987) repeated the experiments of Pierce and Bauer (1983) and stated that the regulatory response of plants doubly inoculated with *B. japonicum* strain 1-110 ARS was not as dramatic as previously reported.

While it has been shown above that a 24 hour delay in inoculation of split root grown (*G. max* cv. Bragg) suppresses nodulation of the second root, it is important to note that the autoregulation signal is not necessarily elicited in that first 24 hours. However, what can be accepted is that the first inoculated site has 24 hours head-start on infection and nodule development, and so when the infection of the first site reaches a certain stage in development (whether it be 24 hours, 48 hours or 7 days), it will be in a more advanced position to signal the autoregulation of nodules on the opposite root. Thus it must not be assumed that the autoregulation signal is elicited in the first 24 hours after inoculation.

Regulation of nodule number per plant

Control experiments in which side 1 was inoculated at time zero and side 2 was left un-inoculated confirmed the sterility from one side of a split root system to the other as no nodules developed on side 2. Interestingly, more nodules formed on side 1 of this system than on side 1 of the system in which both sides were inoculated at time zero. Indeed the total nodule number per plant was equal for the Early / Early split root system and the Early / Un-inoculated treatment. This agrees with the results of Bohlool *et al.* (1986) who stated that under nitrate free conditions (as in Table 5.1) the total number of nodules per plant (wild-type) tends to remain constant regardless of whether 1 or both sides are

inoculated, whereas on nts382 plants there were twice as many nodules when both half root systems were inoculated. However, total nodule mass remained constant for both plant types, indicating that while nts382 does not regulate infections, nodule development is ultimately regulated by plant growth.

Sucrose partitioning in split root systems of cv. Bragg

Experiments by Singleton and van Kessel (1987) have suggested that soybean selectively partitions dry matter to the source of N_2 fixation. They showed that in a split root system in which the gaseous environment of each half root was controlled (and hence the nitrogen fixation ability of the nodules), that growth of nodules and roots on the air side of an air/ArO_2 split root system was significantly greater than on the ArO_2 side and significantly greater than root and nodule growth of air/air plants. They indicated that root stimulation is the result not of infective interaction between *Bradyrhizobium* and the host but is due to the products of nitrogen fixation.

They also showed that current photosynthate ($^{14}CO_2$) was selectively partitioned to the source of fixed N_2. Partitioning of ^{14}C labelled photosynthate was a function of both the size and intensity of the below ground sink created by N_2 fixation.

However data in chapter 5 shows that Bragg split root weights remained equal regardless of whether 1 or both sides were inoculated (and fixing N_2). The disparity could stem from the fact that these experiments were performed in the presence of 0.5 mM KNO_3 whereas Singleton and van Kessel had nitrate free conditions.

It must be noted that the ^{14}C translocation experiment can only indicate the ^{14}C content of the roots at the moment of harvest and thus it is possible that the overall flux of ^{14}C (and hence metabolism) to the first inoculated root was greater than that of the delayed side. Future experiments involving a time course of ^{14}C- sucrosetranslocation at regular intervals from inoculation to harvest would clarify this.

Effect of cultivar of the autoregulation response

Three nts mutants (nts1116, 1007, 382) as well as the wild type cultivar Bragg showed no significant difference in the partitioning of fresh root weight despite a 7 day delay in inoculation of the second side when grown in the presence of low level nitrate. This was also observed in nts382 plants grown in the absence of nitrate. In contrast two of the wild type cultivars, namely Clark and Williams, preferentially allocated root weight to the first inoculated side. When Bragg roots were grown in the absence of nitrate the partitioning of root weight tended towards the direction of the early inoculated root system. The preceeding discussion of root weight allocation was written within the

guideline of statistical significance. However, another approach can be taken in which one looks at the trends rather than wholly adhering to statistics. For example, though the differences were not significant, in Tables 5.2 and 5.4 (+ N) the root half receiving the primary inoculum had consistently higher root weights than those receiving delayed inoculations. In Table 5.1 (- N), Bragg half root systems were consistently (and significantly) larger on the nodulated sides. In Table 5.5, in all of the cultivars except nts382, root weights were higher on the side with more (primary) nodules, although the differences were significant for only two cultivars (Clark and Williams). Given these conclusions, then, these results support Singleton and van Kessel's (1987) conclusions that the plants selectively partition resources to localized sources of N assimilation. Experimental resolution of these two hypothesis is complicated by the fact that nodulation is scored after several weeks. This means that processes governing nodule initiation, nodule growth and nitrogen fixation become inter-related and direct causative relations are difficult to ascertain. Perhaps further work perhaps using nodulation detection after 2 or 3 days may help to resolve the above (for technique see Calvert *et al.* 1984).

The application of the split root system, delayed inoculation and the use of genetic variability as shown here demonstrates that systemic communication between different plant parts is significant in the control of nodulation.

CHAPTER SIX

CONCLUSIONS

The present understanding of the regulation of nodulation in the *Bradyrhizobium*-soybean symbiosis has been made possible through the application of a variety of techniques taken from plant physiology, molecular biology, cell biology, genetics (of both the macro and micro symbionts) and chemistry. The aim of this thesis has been to incorporate results from all of these fields into a general model of soybean nodule autoregulation. In particular this study has investigated the interactions of trans-acting factors (e.g. phyto-hormones and nutrients) as well as *nod* gene products to initiate gene regulation and its phenotypic expression as often demonstrated by developmental changes.

Host regulation of the soybean-*Bradyrhizobium* symbiosis, in terms of the number of initiated nodules, and their subsequent level of development is controlled, in part, by internal factors in a process referred to by Carroll *et al.* (1985a) as "autoregulation" of nodulation. Additionally, nitrate has been shown to inhibit the nodulation process. Previous investigations of the autoregulation response however have been confused, owing to artifacts of the experimental technique, caused by nitrate deficiencies and limited growth conditions.

Original work by Bhuvaneswari *et al.*(1980) suggested a feed-back mechanism of control that operated with-in two hours of inoculation, but this time span was later extended to 10 -15 hours by Pierce and Bauer (1983). However, both these experimental findings need to be interpreted within the context of the experimental design. Soybeans were grown in plastic pouches lined with moistened absorbant paper and limited tap root growth to approximately 15 cm. Further, the lower portion of each root may have been subjected to water-logging, and hence oxygen deprivation, since the nutrient solution tended to collect in the bottom of each pouch. While growth pouch analysis may be useful for short term infection experiments, it seems unlikely that pH, oxygen concentration and nutrient supply can be maintained for the longer time required for nodulation studies. As such, the 10-15 hour inhibition of nodulation may have been an artifact of the system. Indeed, Heron and Pueppke (1987) were unable to repeat quantitatively, Pierce and Bauer's (1983) findings when similar conditions were employed. Hence the concepts developed by Bauer's laboratory need to be judged more along the qualitative, rather than the empirical message.

Singleton (1983) and Kosslak and Bohlool (1984) developed an alternative reliable technique for examining the autoregulation response. They grew soybeans in nitrogen free split root systems and, by inoculating the first side and then delaying the inoculation of the second side, showed that significant suppression of nodulation on the second side could be demonstrated after 96 hours, and total suppression after 10 days. They

suggested that control by the soybean host over the number of sites leading to successful infections appears to be executed during the early stages of the infection process and is also regulated by the amount of light available to the host for photosynthesis.

Their explantation however, may be misleading due to limitations of the experimental design. The root of the first inoculated side was often larger than the delayed side, and so the suppression may have been enhanced by the the first inoculated side responding to the inoculant dose and drawing more assimilates in a similar way that plant roots respond to nutrient supply, as well as via a systemically translocated autoregulation signal. As the growth system was watered with a nitrate free nutrient solution, it is possible that nodulation of the first side utilized the limited supply of assimilates, and thus deprived the second side of metabolites required for growth, thereby inhibiting nodulation.

The experiments in chapter 5 resolved this situation, by providing split root systems with either a small non-inhibitory level of combined nitrogen (0.5 mM KNO_3) in the nutrient solution, or none at all. In addition, a study of the autoregulatory mutant, nts382 was included.

In Bragg significant suppression of nodulation on the second side was observed when inoculation (*B.japonicum* strain USDA110) was delayed for 24 hours, and total suppression was observed when inoculation was delayed for 7 days. No significant difference was observed between the root fresh weights on either side. In a repeat of Kosslak and Bohlool's (1984) experiment nitrate was omitted from the nutrient solution and while total suppression was manifested after 7 days, the root weight of the first inoculated side was consistently higher than the second side.

The requirement for a low level of nitrate was further emphasised with the results from the nts382 mutant. In the absence of nitrate a 7 day delay in inoculation resulted in a 78 % suppression of nodulation, and a smaller root system compared to the first inoculated side. However, in the presence of low level nitrate no such nodule suppression was observed, and neither was there a difference in root weights. This confirms that the nts382 suppression in the former case (minus nitrate) was not due to the presence of an autoregulation signal, but rather, due to a limitation of assimilates on the delayed side.

It is proposed here that the autoregulation signal is induced 24 hours after the initial inoculation and that by day 7 after inoculation, the initial signal has orchestrated a total suppression of further nodulation. This is substantiated by the fact that under similar conditions nodule arrest occurs at the stage of nodule emergence rather than at the stage of root hair infection (Matthews 1987; Calvert *et al.* 1984). Further, it demonstrates that

nitrate starved plants will translocate metabolites and photosynthate to the first inoculated side.

Most importantly, these experiments demonstrate that autoregulation is caused by a systemic response, and is not a function of root size, since nodule suppression occured in split root systems where both root weights were equal. Further, when ^{14}C-sucrose was injected into the shoots of autoregulated Bragg split root systems (nitrate grown) there was no significant difference in the amount of sucrose translocated to either root. This contrasts to results from Singleton and van Kessel (1987) who stated that in split root systems, the amount of dry matter and current photosynthate was greater in fixing than non-fixing roots. Their data must be interpreted carefully, as their experiments were performed in the absence of nitrate, and so may relate to plants experiencing nitrogen stress and related physiological changes.

The discovery of nitrate tolerant symbiosis mutants (Carroll *et al.* 1985 a,b) which developed exceptionally high nodule numbers, coupled the two processes of autoregulation and nitrate inhibition of nodulation. This view was also shared by Malik *et al.* (1987) who stated that nitrate inhibition provides evidence for a host regulatory mechanism that controls the establishment and functioning of the symbiotic association in relation to the supply of fixed nitrogen in the soil. A paradox arose, in that the nitrate effect was shown to be localized (Hinson 1975; Carroll and Gresshoff 1983), while others (Delves et al. 1986; Matthews 1987; this thesis) suggested that autoregulation was a systemic process.

Further evidence linking the action of nitrate to the autoregulation process was derived from approach graft experiments with nts382 and Bragg as shown in chapter 3. It was shown that Bragg: nts382 heterografts grown in the absence of nitrate showed no change in their respective nodulation patterns compared to homograft controls. However, in the presence of 5.5 mM KNO_3 Bragg nodulation was increased 8 fold and nts382 nodulation was suppressed 3 fold. This not only shows that nitrate can trigger (or accentuate) the autoregulation signal but also that the signal can be systemically translocated across the graft junction. The change in nodule numbers was attributed to a dilution effect with one "dose" of autoregulation signal being shared between the two roots. These data extended previous reports by Delves *et al.* (1986), who showed that a systemic factor was involved in autoregulation (see table 3.1). However, they were unclear as to whether autoregulation was caused by an inhibitory signal in the shoots of Bragg plants, or whether there was a nodule enhancing factor in the shoots of nts382 which caused super-nodulation.

Data from wedge graft experiments using challenged and un-challenged plant parts resolved this question by demonstrating that the autoregulation factor is present as an inhibitory signal in the shoots of Bragg plants, while the nts382 mutant is altered in the autoregulation pathway such that it demonstrates a lowered capacity to trigger the autoregulation signal in the presence of inoculum and nitrate. The potential ability of the signal molecule to cross the graft junction equally well in the presence or absence of nitrate was demonstrated by ^{14}C 2,4-D translocation discussed experiments in chapter 3.

The regulation of nodule number via a feed-back mechanism in existing nodules was proposed by Nutman (1952) who showed that the removal of developed nodules from red clover plants stimulated a transient increase in nodule development. This suggested that a nodule inhibitor was present in existing nodules, such that the removal of nodules, and hence inhibitor, allowed further infection events to occur.

Grafting experiments in chapter 3 provide further information about the mechanism of the feed-back control in nodule regulation, but suggest that it occurs in a slightly different manner. It is here proposed that the feed-back mechanism of control operates only to suppress the development of new nodules once a prohibitive level of infections has occurred. When the signals (possibly hormones) which are produced at each infection site become concentrated enough, they elicit changes in shoot tissue, which eventually result in the production of the autoregulation signal. This signal is then systemically translocated to the root to prevent the development of sub-epidermal cortical cell divisions into nodules. There is no evidence to suggest that non-nodulated root tissue can signal the shoots to halt the production of autoregulation signal via a feed-forward mechanism. This proposal is supported by the results from the Bragg challenge experiment (section 3.7). In this experiment, Bragg plants which were inoculated at planting, initiated the usual infection events and nodule development pattern. The shoots of these "challenged" plants were removed either 10, 12,15 or 18 days after planting and immediately grafted onto similar age, but un-inoculated (un-challenged) Bragg roots. The entire graft was subsequently re-inoculated. At harvest, (day 39 after planting) it was shown that the longer the shoot remained intact prior to grafting (i.e. the longer the period of challenge), the greater the suppression of nodulation. In contrast, control plants which used un-inoculated (i.e. un-challenged) shoots demonstrated no suppression of nodulation.

This suggests that the initial Bragg root infection signal (from intact challenged plants) initiates changes in the shoot physiology and biochemistry such that an autoregulation signal or substance is produced. This signal is systemically translocated and graft transmissible, and causes suppression of nodulation. Further, it is not over-ridden by signals in un-inoculated roots. In essence, the shoot behaves as if it is still attached to its

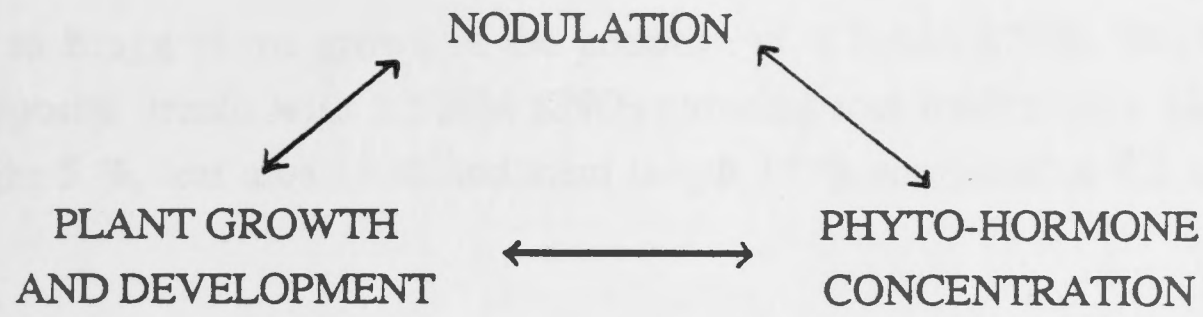
previous well nodulated root and continues to produce autoregulation signal. The reason for this strong suppression of nodulation on newly inoculated roots could be that the concentration of shoot derived autoregulation signal stays high for a long period of time (at least 18 days in this experiment) because it is catabolised slowly. Thus when the challenged Bragg shoot is grafted onto the newly inoculated root it translocates a high level of signal which is prohibitive to nodule development.

Nitrate may therefore act in a similar manner as this acropetal (i.e. root to shoot) feed-back mechanism by stimulating the synthesis and translocation of infection signals or by mimicking the infection signals thus promoting the conditions required for autoregulation. Alternatively, nitrate may either enhance the production or action of the basipetal (shoot to root) signals thus eliciting or enhancing the autoregulation response. This would accounting for nitrate inhibition of nodulation and could occur directly, or via interaction with pre-existing phyto-hormones in the infected zones of the root.

Previous studies (outlined in chapter 4) have shown that nodules, and infected root tissue, are a rich source of plant hormones. It has been suggested that hormones are involved in the production and movement of assimilates to developing organs (Thomas 1986). For example, Goodwin *et al.* (1978) stated that the nutritional status of the plant, particularly the supply of nitrate, may significantly affect root derived cytokinins and, consequently, shoot development and physiology (see chapter 4). Further, Trevawas (1982) suggested that well nourished plants have a greater rate of protein synthesis, and hence number of cells, than mal-nourished equivalents.

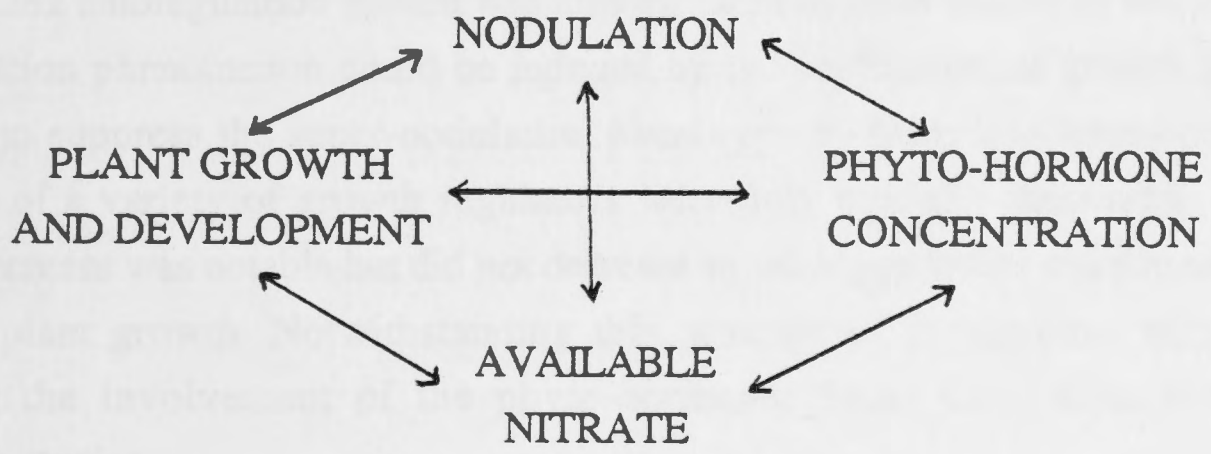
As hormone receptors are most likely to be proteins the addition of nitrate to split root systems may increase the plant receptor density and allow cells to divide for longer periods of time. Thus, roots on both sides of the split root system could develop equally well regardless of their nodulation patterns. Conversely, mal-nourished plants may have a lower hormone receptor density. The first inoculated side would therefore draw a greater proportion of the limited structural resources (e.g. photosynthates, amino acids), leaving the second inoculated side depleted. This would result in not only a smaller root, but also a suppression of nodule number. This is why the addition of a low level of nitrate in the nutrient solution is considered an important part of the experimental set-up.

The involvement of phyto-hormones in autoregulation has been suggested for the last 30 years, however little conclusive evidence can be drawn from the range of phyto-hormone studies since, in many cases, the application of hormones not only generated changes in nodule number and development but also generated changes in plant growth and development. There is a triangular relationship between nodulation, phyto-hormones and plant growth in which each affects the other as represented overleaf:



Thus, it is often difficult to discern whether applied hormones or growth regulators are having a direct effect on nodulation or whether nodule suppression is an indirect consequence of reduced plant growth and development. Reported experiments have often disregarded this concept and have stated that a particular hormone suppresses nodulation without investigating the changes in plant growth. Therefore this study has usually included data for nodule, root and shoot weights and, where necessary, expressed nodule number as a function of plant growth.

Interpretation of results obtained from phyto-hormone application studies is also complicated by the fact that the level of available nitrate can alter the nodulation response (in particular see IAA results). Thus the triangular model can be further expanded to show the effect of nitrate in the plant system as follows:



The presence of nitrate in the growth system has the potential to alter nodulation either directly (e.g. through interference with infection events), or indirectly via changes in endogenous phyto-hormone levels or through variations in plant growth and development, perhaps through changes in hormone receptor density or sensitivity.

The results from chapter 4 (Table 4.12) show that nitrate can be assimilated from the roots and act systemically throughout the plant to bring about changes in plant physiology and biochemistry. Bragg plants grown in the presence of 5.5 mM KNO_3 increased root fresh weight 104 %, shoot fresh weight 40 %, leaf area 30 % and stem length 17 %

compared to Bragg plants grown in the presence of 0.5 mM KNO_3. Nts382 plants showed opposite trends with 5.5 mM KNO_3 reducing root fresh weight 12 %, shoot fresh weight 5 %, leaf area 18 % and stem length 17 % compared to 0.5 mM $KN0_3$ controls.

However, the higher level of nitrate reduced Bragg nodule number, nodule fresh weight and specific nodule weight by 55 %, 63 % and 17 % respectively. Conversely, nitrate increased these parameters in nts382 by 28 %, 64 % and 28 % respectively. This demonstrates that nitrate not only has a localized effect, as exemplified by suppression of nodulation, but also that nitrate can generate changes in whole plant physiology. Thus it must be emphasized that while nitrate may act in a localized fashion to bring about nodule suppression (through interference with infection events), some of the suppression may be attributed to changes in whole plant physiology and biochemistry.

The results presented in this thesis are an extension of early phyto-hormone studies, to investigate the interaction of nitrate with exogenously applied phyto-hormones and their combined effect on nodulation was investigated. It was attempted to clarify the conflicting information generated from experiments ranging over three decades, in which there was often no consistency between plant species, phyto-hormone concentration, duration, mode, and location of application, or age of plant material.

The nts382 autoregulation mutant was also included in these studies to see whether the autoregulation phenomenon could be induced by the application of growth regulators. Attempts to suppress the super-nodulation phenotype by foliar and inter-cotyledonary injections of a variety of growth regulators were only partially successful, as nodule number decrease was notable but did not decrease to wild-type levels and patterns without affecting plant growth. Notwithstanding this, a series of conclusions may be made regarding the involvement of the phyto-hormones IAA, GA_3, GA_4 and ABA in nodulation studies.

Wild-type soybean, grown on high level nitrate (5.5 mM KNO_3) was insensitive to daily inter-cotyledonary injections of indole acetic acid (1-10 μg), with no change in nodule number or total nodule weight being demonstrated. In contrast, the similarly treated nitrate tolerant, autoregulation mutant nts382 demonstrated a significant suppression of nodule number and weight. Although the suppression of nodulation was not to wild-type levels, it is still evident that this auxin may be involved in the stimulation of, (or can mimic), the autoregulation response.

Although IAA (at tested concentrations) had no effect on Bragg nodulation, daily injections resulted in a significant reduction in root fresh weight at all tested

concentrations. This agrees with previous observations (see section 4.1.2) that auxin inhibits the elongation of roots. It has been previously stated that the suppression of nodulation by IAA (on *Phaseolus vulgaris*) is a secondary response due to the reduction of root elongation, and subsequently a reduction in the number of sites available for nodule formation (Cartwright 1967). However, nts382 plants, when treated with IAA (1-10 μg), demonstrated nodule suppression while showing no change in root fresh weight. Additionally, nts382 stem length was significantly increased to equal that of Bragg plants. Thus, the addition of IAA to nts382 plants stimulated the elongation of stem length, which is a phenomenon often elicited by increased gibberellic acid synthesis or application. It is thus a possibility that the application of IAA to nts382 triggered a response, involving gibberellic acid to bring about the suppression of nodulation.

The involvement of gibberellic acid in the autoregulation process was further investigated through the use of daily inter-cotyledonary injections of GA_4. Bragg plants grown in the presence of low level nitrate (0.5 mM KNO_3) demonstrated no change in nodule number or total nodule fresh weight after treatment with daily injections of 10 ng to 5 μg GA_4. Nts382 plants grown under the same conditions demonstrated significant suppression of nodule number. Total nodule fresh weight was not reduced significantly and specific nodule weight remained the same as untreated controls, showing that GA_4 suppresses nodule number of the autoregulation mutant but has no real effect on the development of existing nodules.

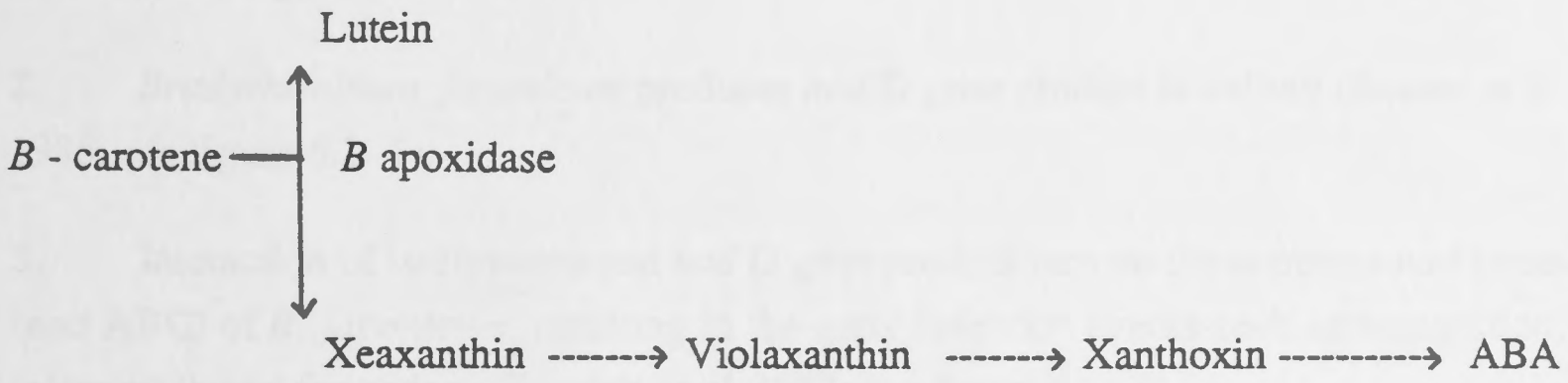
The application of GA_4 had no effect on the shoot fresh weight of Bragg plants but significantly reduced that of nts382 plants. However, when nts382 nodule number was expressed per gram of shoot fresh weight there was still a significant reduction in nodule number, showing that the reduction in shoot weight was not the sole cause of the suppression. These results are not consistent with those obtained from Williams and de Mallorca (1984) who showed that daily foliar applications of GA_4 (3.01×10^{-5} M) delayed the formation of nodule initials and reduced the numbers, nodule mass and specific nitrogenase activity of *G. soya* cv Jupiter. This discrepancy could be due to the absence of nitrate in the nutrient solution, or to the fact that nodulation tests were performed in plastic growth pouches which, as previously disclosed, may interfer with the nodulation response.

The conversion of GA_4 to GA_3 in *Gibberella fujikuroi* has been previously established (Fletcher *et al.* 1958), but the intermediacy of GA_4 in higher plants has not yet been demonstrated. Regardless of this however, GA_4 mimics the wild-type autoregulation response when applied to the nts382 mutant.

The involvement of abscisic acid in nodule regulation was tested via daily injections into Bragg and nts382 cotyledons immediately after inoculation. Bragg plants grown in the presence of 5.5 mM KNO_3 demonstrated significant nodule suppression as a result of 100 ng daily injections, but nts382 plants grown under the same conditions did not display significant nodule number suppression until the concentration was increased to 5 μg per injection. Thus, nts382 is 50 times less sensitive to nodule number suppression than Bragg.

Although the above results strongly implicate the involvement of phyto-hormones in the autoregulation process, it must be considered that the mechanisms which suppress nodulation of nts382 plants after the application of exogenous hormones may only be mimicking the actual autoregulation response observed in its wild-type parent cultivar. However, data from studies of changes in the concentration of endogenous phyto-hormones after inoculation also suggest that autoregulation involves alterations in intermediates in the ABA pathway.

In particular, Krotzky *et al.* (pers. comm from Dr. A. Krotzky) demonstrated that nts382 plants grown in the presence of 0.8 mM KNO_3 contained only 20 % as much endogenous ABA as similarly grown Bragg plants. The reduced level of ABA in nts382 may be due to a mutation in the anabolic pathway to ABA or alternatively, the catabolism of ABA in nts382 could be altered such that catabolism is so fast that ABA intermediates are in low concentration. However, this latter possibility is not as likely, since high speed catabolism would probably stimulate anabolism. The former explanation is also supported by the observation by Eskins and Harris (1981) who indicated a variation in the pathway from *B*-carotene to ABA which contains a regulatory step, as shown below (refer also to figure 4.2):



In this pathway, *B*-carotene may be converted to either lutein or xanthoxin, with the ratio of these two products being regulated by a *B* apoxidase enzyme. Results of Krotzky

et al. (pers. comm. from Dr. A. Krotzky) provided evidence for a higher ratio of lutein to xeaxanthin, as well as severely reducing the quantity (35 %) of violoxanthin in nts382 as compared to Bragg. Additionally they showed that the level of violoxanthin in nts382 was approximately 35 % of that observed in Bragg plants. Thus, it is proposed that nts382 is less sensitive to nodule suppression by ABA than Bragg because a mutation in the enzyme controlling the ratio of lutein to xeaxanthin causes a preference for lutein production. This would result in either (1) the anabolism of zeaxanthin being slower in nts382 (and hence violoxanthin concentration) than Bragg or (2) the catabolism of zeaxanthin being faster in nts382. Either way, nts382 would require a greater concentration of applied ABA to suppress nodulation than would Bragg.

The results from GA_3 application studies showed the opposite trend to that of the ABA data. In particular it was observed that the nts382 mutant was more susceptible to nodule suppression by daily foliar applications of GA_3 (2.89×10^{-6} M) than was Bragg since conditions that had no effect on Bragg nodule number significantly reduced that of nts382 plants. This indicates than nts382 plants have a higher endogenous level of gibberellic acid than Bragg such that further application suppresses nodulation. Since the gibberellic acid and abscisic acid pathways have mevalonic acid as a precursor, it is possible that the balance of GA and ABA is involved in the manifestation of the autoregulation response. If nts382 is altered at the lutein / zeaxanthin position in the ABA pathway it is possible that the ratio of the end-products of these two pathways in nts382 is tipped towards gibberellic acid anabolism. This would explain why nts382 is less sensitive to ABA inhibition, and more sensitive to GA inhibition of nodulation.

The conclusions of this thesis may be incorporated into a general model of nodulation and autoregulation, as seen in the wild-type soybean plant, as follows:

1. Soybean roots exudates contain the isoflavones daidzein and genistein (Kosslak *et al.* 1987; see figure 6.1 -1).

2. *Bradyrhizobium japonicum* produces *nod* D gene product in culture (Rossen *et al.* 1985; see figure 6.1 -1).

3. Interaction of isoflavones and *nod* D gene product turn on the common *nod* genes (*nod* ABC) of *B. japonicum*, resulting in the early infection events such as recognition, infection thread formation (Kosslak *et al.* 1987; see figure 6.1 - 2).

4. Inoculation of soybean roots induces cortical cell divisions, even before infection occurs (Calvert et al. 1984; Bauer *et al.* 1985; see figure 6.1 - 2).

Figure 6.1 **Proposed model of nodule autoregulation in the *Bradyrhizobium* - soybean symbiosis**

1. Soybean roots produce root exudate which contains the iso-flavones daidzein and genistein.

   Bradyrhizobium japonicum express *nod* D product in culture.

2. Iso-flavones and *nod* D gene product interact and turn on the common *B.japonicum nod* genes ABC.

   Sub-epidermal cortical cell divisions occur in the presence of *B.japonicum*.

3. Cortical cell division / infection factor (possibly phyto-hormonal) is produced by dividing cortical cells.

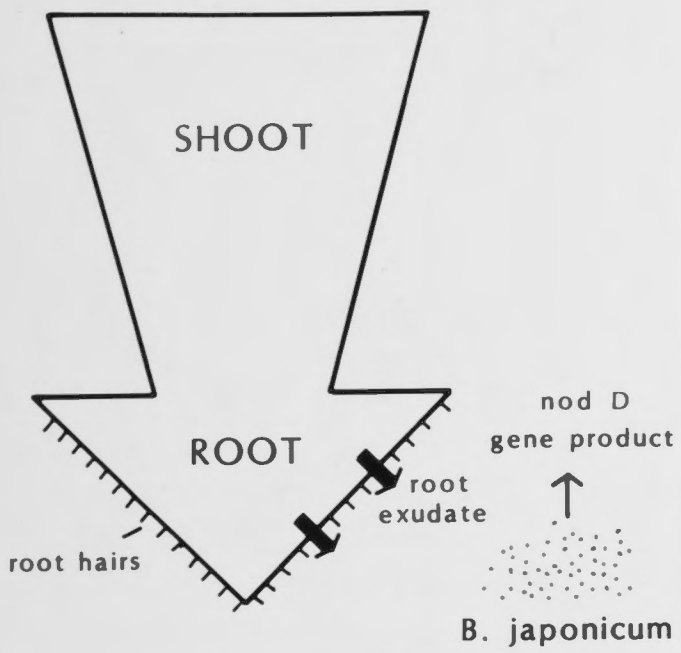
4. Cortical cell division factor (or a hormone stimulated by it) binds to specific hormone receptor sites (R_O) in the shoots and forms a hormone - receptor complex (R_1).

   This may result in an increase in hormone sensitivity and changes in the properties of receptor proteins.

5. Changes in properties of R_1 may result in the production of signals (S_O and S_1) which affect transcription processes, or changes in R_1 may result in alterations of phyto-hormone balances (e.g. GA : ABA).

6. Increase in ABA concentration (or related signal) blocks the development of further cortical cell divisions, thus causing nodule suppression or autoregulation.

1



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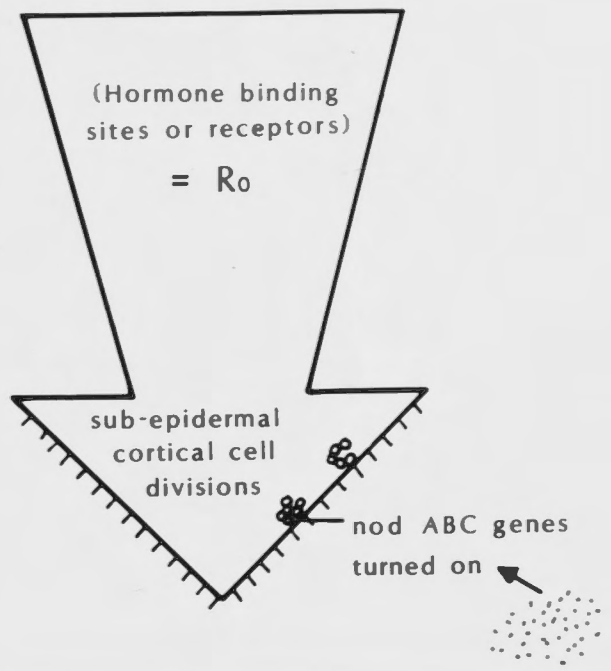

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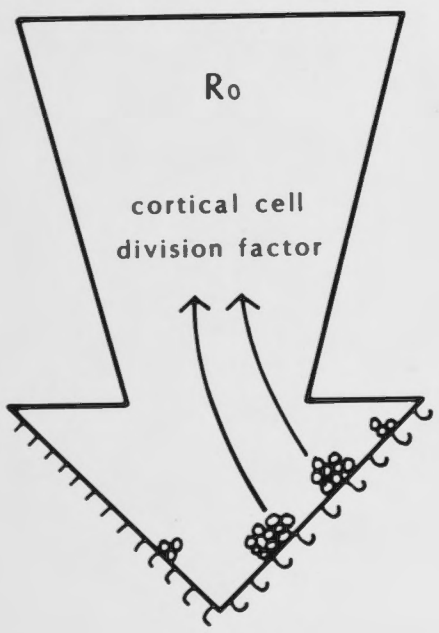

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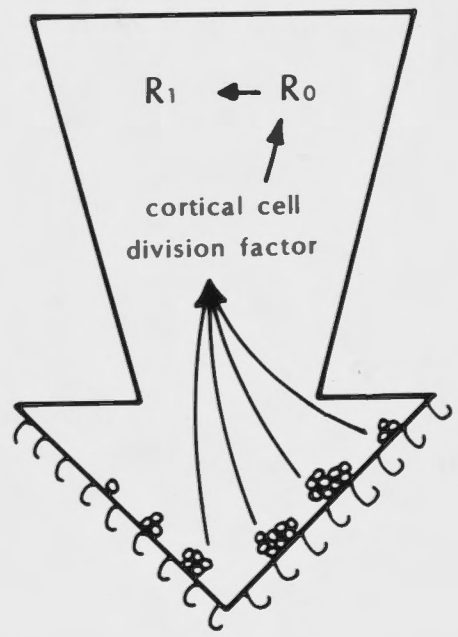

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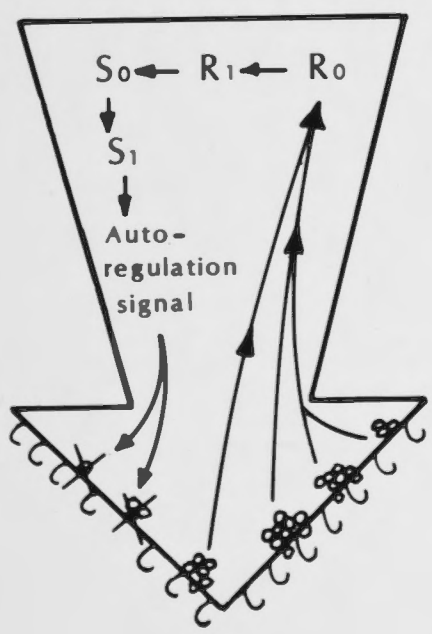

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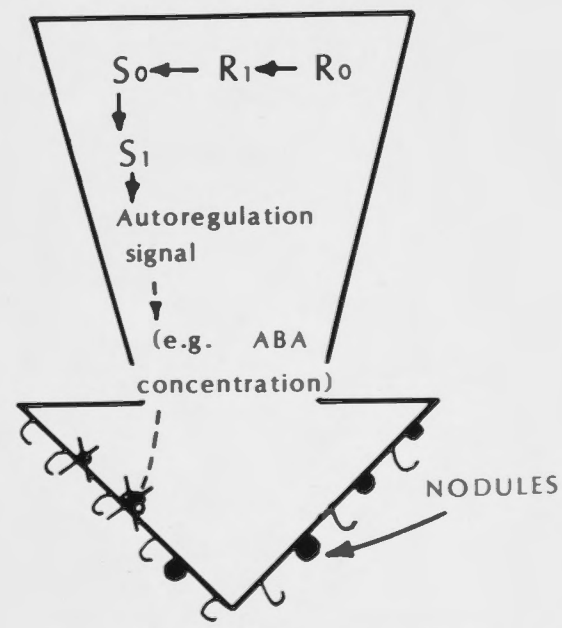


High ABA concentration arrests further cortical cell division therefore SUPPRESSES NODULATION

5. Phyto-hormones such as auxin and cytokinins are required for cortical cell division, and as such each infection event that reaches cortical cell division stage produces a low concentration of phyto-hormone (Das *et al.* 1956; Matthysse and Torrey 1967; Phillips and Torrey 1970; Black and Hamilton 1976; Bauer *et al.* 1986; see figure 6.1 - 3).

6. These phyto-hormones or associated signals are translocated or transmitted throughout the plant. In the shoot, these phyto-hormone signals or chemical messengers enter the cells and bind to specific receptors and form hormone-receptor complexes. The result is an increase in hormone sensitivity, and a change in the properties of the receptor proteins (see also Trevawas 1981,82,83; Bruinsma 1985; Hall 1987; Hall and Thomas 1987; see figure 6.1 - 4).

7. The concentration of cortical cell division factor is correlated with the number of infection events on the root. When the concentration becomes high it changes the hormones sensitivity of the receptor complex, thus communicating to cells in the leaves that a sufficient amount of infection events have occured (see figure 6.1 - 4)

8. Changes in the properties of receptor proteins in the leaves may result in the production of a signal which travels to the nucleus (or other DNA containing organelle) to specifically affect the transcription process. In addition the hormonal signal may affect the translation of mRNA resulting in the activation of particular genes and enzymes. Alternatively, the concentration of cortical cell division factor may change the ratio of endogenous hormones (e.g. GA and ABA) and bring about changes in transcription in a similar manner (see figure 1.6 - 5).

9. The shoot derived signal(s) is / are systemically translocated and act(s) to suppress the development of newly initiated cortical cells divisions into nodules (see figure 6.1 - 6).

10. In addition the autoregulation signal is:

(a) initiated 24 hours after inoculation (Chapter 5),

(b) systemically translocated (Chapters 3 and 5),

(c) graft transmissible (Chapter 3),

(d) long lasting and slowly catabolised, and therefore remains active in the plant for at least 18 days after inoculation (Chapter 3),

(e) cannot be turned off, or over-ridden by nodule feed-back, (Chapter 3),

(f) accentuated or mimicked by nitrate (Chapter 3,4 and 5).

11. The autoregulation phenomenon is possibly caused by an alteration in the GA / ABA pathway such that a higher concentration of ABA is produced after infection (or high nitrate application) (Chapter 4), and interfers with the development of cortical cell divisions required for nodule formation (i.e. supporting results from Phillips 1971, as outlined in 4.1.3.2).

12. Nitrate is assimilated throughout the plant and therefore may act systemically to alter the hormone susceptibility of receptor sites throughout the plant. (i.e. nitrate makes the whole plant, and not just the root more susceptible to the autoregulation [phyto-hormone] signals) (Chapter 4). Additionally, nitrate may stimulate either the acropetal (root to shoot) cortical cell infection signal, or enhance or mimic the basipetal (shoot to root) autoregulation signals to accelerate the autoregulation response (see figure 6.2)

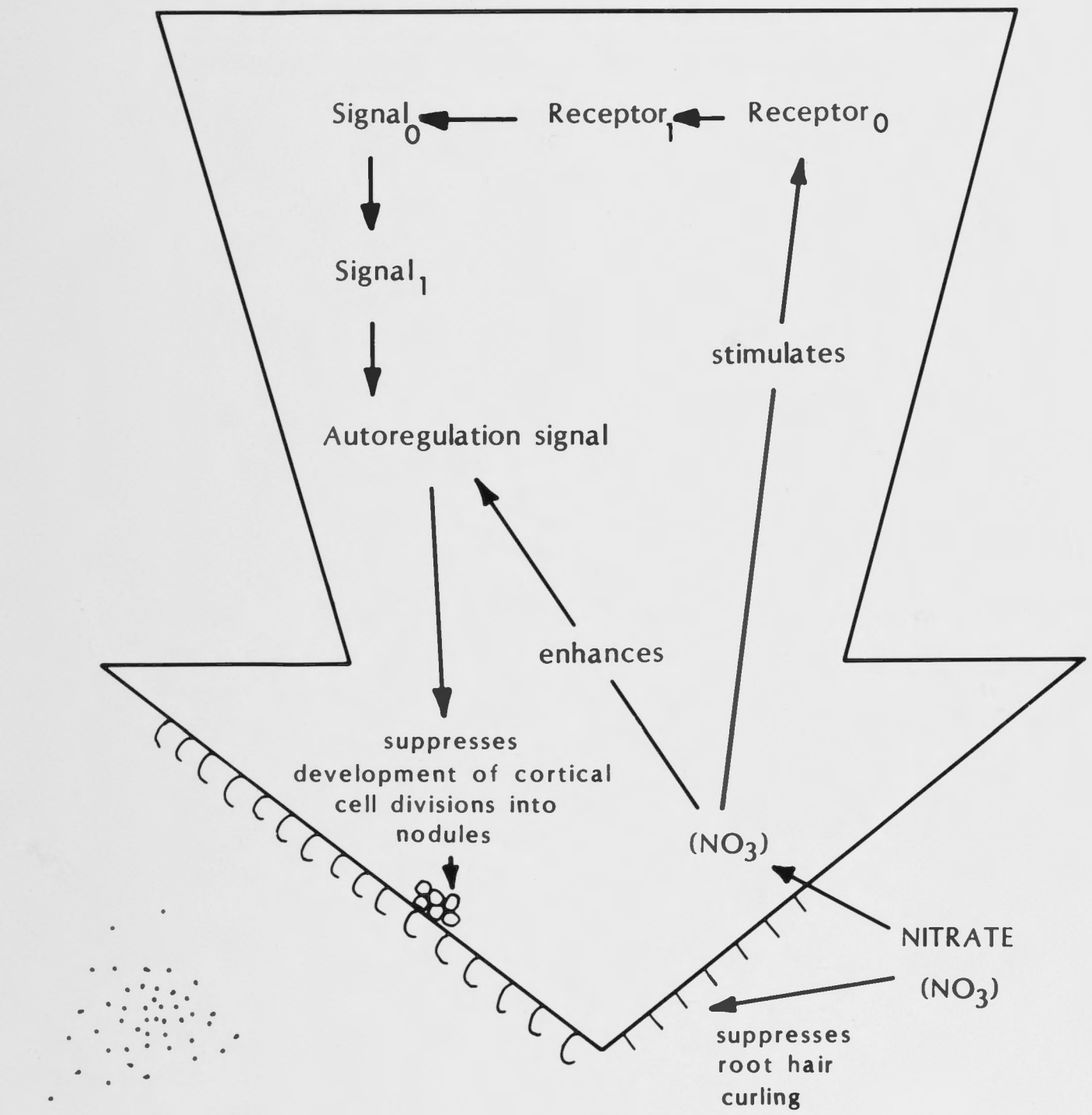
This thesis has provided an initial characterization of the nodule autoregulation mechanism in the wild-type soybean *Glycine max* (L.) Merr cv. Bragg. In addition, the preceeding models propose an explanation for autoregulation mutant nts382 which results in a super-nodulation and nitrate tolerant phenotype. The final identification of the autoregulation signal will undoubtedly be made through investigations of the changes in endogenous signals in the shoot tissue, rather than by attempting to correct the nts382 phenotype with external applications of phyto-hormones or intermediates. A preliminary study described in chapter 4, using HPLC analysis of labelled mevalonic acid incorporated into shoot tissues shows that there are differences in the eluent profiles between Bragg and nts382 plants. These differences may have nothing to do with autoregulation, but their identification (by comparison with known standards) is still of great interest. One could re-feed the fraction(s) exclusive to the Bragg (autoregulated) shoot back into the nts382 plant to attempt the correction of the mutant phenotype. If this procedure worked and nts382 nodulation was suppressed to wild-type levels without affecting plant growth, then the study could be expanded to investigate the timing of the synthesis and the nature of the suppression signals. The interaction of nitrate with the autoregulation signal could be tested in a similar manner.

Figure 6.2 **Proposed model of nitrate inhibition of nodulation in the *Bradyrhizobium* - soybean symbiosis**

1. NO_3 may directly suppress root hair curling.

2. NO_3 may stimulate changes in hormone receptor sensitivity (R_O) therefore allowing cortical cell division factors to act at lower concentrations.

3. NO_3 may directly enhance or mimic the autoregulation signal.



Bradyrhizobium japonicum

Appendix 1: Effect of daily foliar application of GA_3 and CCC on nodulation and plant growth (Data expressed as the percentage stimulation or inhibition as compared to the 100% control) (Experiment 1)

Treatment	Nodule Number	Nodule Dry wt. (g)	Root Dry wt (g)	Shoot Dry wt. (g)	Total Dry wt. (g)
382 + N	100%	100%	100%	100%	100%
382 + N + CCC	169	132	118	122	120
382 + N + GA_3	40	40	65	98	89
B + N	100	100	100	100	100
B + N + CCC	373	200	159	147	120
B + N + GA_3	68	60	57	92	89
382 - N	100	100	100	100	100
382 - N + CCC	173	126	220	133	141
382 - N + GA_3	67	96	167	125	127
B - N	100	100	100	100	100
B - N + CCC	137	118	192	106	101
B - N + GA_3	85	106	184	138	122

+N = Herridge solution supplemented with 5.5 mM KNO_3
- N = Herridge solution without nitrate
CCC = Chloro-choline-chloride
GA_3 = Gibberellic acid
382 = nts382, B = Bragg

Appendix 2: Effect of foliar application of GA_3 and CCC on nodulation and plant growth (Data expressed as the percentage stimulation or inhibition as compared to the 100% control) (Experiment 1)

Treatment	Specific Nodule wt (mg)	Nodule No. / g. nodule dry wt.	Nodule No. / g. root dry wt.	Nodule No. / g. shoot dry wt.	Nodule No. / g. total dry wt.
382 + N	100%	100%	100%	100%	100%
382 + N + CCC	100	193	163	142	137
382 + N + GA_3	120	132	50	41	42
B + N	100	100	100	100	100
B + N + CCC	45	142	312	93	278
B + N + GA_3	48	132	188	29	101
382 - N	100	100	100	100	100
382 - N + CCC	80	148	75	108	121
382 - N + GA_3	140	64	49	50	51
B - N	100	100	100	100	100
B - N + CCC	80	98	111	153	140
B - N + GA_3	110	76	70	67	67

+N = Herridge solution supplemented with 5.5 mM KNO_3
- N = Herridge solution without nitrate
CCC = Chloro-choline-chloride
GA_3 = Gibberellic acid
382 = nts382, B = Bragg

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